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The Biochemistry and Physiology of Poly-beta-hydroxybutyrate metabolism in Methylosinus trichosporium OB3b

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ABSTRACT

The obligate methanotroph, Methylosinus trichosporium OB3b synthesised up to 20 % of its dry weight as poly-beta-hydroxybutyrate when grown in batch culture or nitrogen limited continuous culture on a methanol/ammonium salts medium. Four soluble enzymes associated with polymer metabolism, D(-)-3-hydroxybutyrate dehydrogenase (3-HBD), acetoacetyl-CoA synthetase, beta-ketothiolase and acetoacetyl-CoA reductase have been purified from the crude extract of this organism. Detailed kinetic studies were conducted on each enzyme and their role in the regulation of polymer metabolism was assessed.

3-HBD was purified over 1800-fold and identified as a monomeric protein (M_r = 26,000 daltons). The enzyme catalysed an ordered Bi-Bi reaction mechanism, and was susceptable to product inhibition by both acetoacetate and NADH. Product inhibition studies highlighted the importance of the NADH/NAD couple in the regulation of this enzyme in vivo; inhibition by NADH gave a K_{is} of 35 μM.

Acetoacetate is metabolised to acetoacetyl-CoA by a novel acetoacetyl-CoA synthetase. The enzyme has an absolute requirement for ATP, CoASH, a monovalent cation (K^+, NH_4^+ or Cs^+) and a divalent cation (Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+} or Ca^{2+}) for the activation of acetoacetate, yielding acetoacetyl-CoA, AMP and pyrophosphate. No acetoacetate:succinate CoA-transferase activity was demonstrated in this organism. The low activity (1.14 units (mg protein)$^{-1}$ of acetoacetyl-CoA synthetase suggested that this might be the rate limiting step in the pathway of polymer mobilisation.

Thiolysis of acetoacetyl-CoA to form two molecules of acetyl-CoA is catalsed by a tetrameric (M_r = 145,000 daltons) beta-ketothiolase. The enzyme is subject to CoASH inhibition, which is competitive with respect to acetoacetyl-CoA concentration. The K_m for acetoacetyl-CoA was determined to be 30 x 10^{-6} M. In the condensation function of the enzyme, CoASH strongly inhibits the reaction leading to non-linear Michaelis-Menten kinetics. It was suggested that this was a function of the reaction mechanism of the enzyme and not necessarily any cooperative interaction between the enzyme and CoASH. On the basis of the kinetic data a major role for beta-ketothiolase in the regulation of polymer synthesis was proposed.

The formation of D(-)-3-hydroxybutyryl-CoA, prior to it polymerisation, is catalysed by a dimeric NADP-specific acetoacetyl-CoA reductase (M_r = 46,000 daltons). The enzyme has a K_m of 4.5 x 10^{-6} M and 42 x 10^{-6} M for acetoacetyl-CoA and NADP respectively and is subject to both substrate (acetoacetyl-CoA) and product (D(-)-3-hydroxybutyryl-CoA and NADPH) inhibition. Kinetic studies suggest that the regulation of acetoacetyl-CoA reductase forms part of a concerted feedback inhibition on the activity of beta-ketothiolase in regulating polymer synthesis.

An integrated system for the control of polymer metabolism was proposed as a result of in vivo quantification of CoASH, acetyl-CoA, acetoacetyl-CoA, D(-)-3-hydroxybutyryl-CoA and succinyl-CoA. This suggested that PHB synthesis occurred as a result of overspill metabolism of acetyl-CoA from the TCA cycle. PHB mobilisation was thought to be regulated by the redox state of the cell.

CHAPTER 1: INTRODUCTION

CHAPTER 5: THE PURIFICATION AND PROPERTIES OF BETA-KETOTHIOLASE FROM M. TRICHOSPORIUM OB3B

CHAPTER 6: THE PURIFICATION AND PROPERTIES OF ACETOACETYL-COA REDUCTASE FROM M. TRICHOSPORIUM OB3B

CHAPTER 7: PHB SYNTHESIS AND *IN VIVO* METABOLITE STUDIES IN *M. TRICHOSPORIUM* OB3B

C H A P T E R O N E

I N T R O D U C T I O N

1.1 INTRODUCTION

Poly-beta-hydroxybutyrate (PHB) is an intracellular reserve polyester peculiar to microorganisms, occurring widely in both Gram-positive and Gram-negative bacteria. The presence of PHB is particularly characteristic of nitrogen-fixing species of Azotobacter where PHB may accumulate up to 70 % of bacterial dry weight.

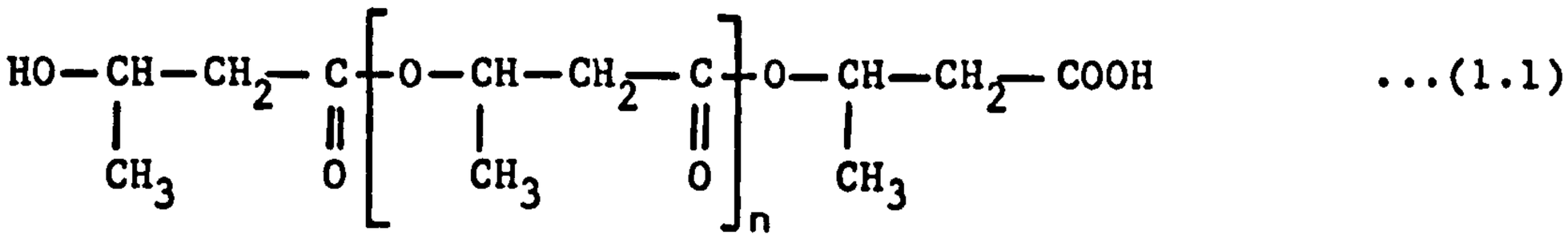
PHB has been known since the beginning of the century (Beijerink, 1901). However, it was not until much later that its chemical identification was described (Lemoigne, 1926). During this early work, Lemoigne isolated two compounds from Bacillus megaterium which were considered to be the hydrolysis products of polymeric beta-hydroxybutyrate. Both compounds had the same empirical formula (C_2H_3O) but differed in their physical properties; one was crystalline (m.p. 120°C) while the other was shown to be amorphous (m.p. 157°C). The latter compound was considered to be PHB.

Over the intervening period, research on aspects related to PHB has been sporadic and it is only in the last twenty years that detailed analysis of its metabolism and biochemical regulation have been made. In this introductory chapter the literature will be reviewed to the present day on all aspects relating to the properties of PHB and its metabolism. Since this thesis is related specifically to a study on PHB metabolism in an obligate methanotroph, a general review of the relevant methanotrophic literature is also included.

1.2 PROPERTIES OF PHB

1.2.1 PHYSICAL AND CHEMICAL PROPERTIES

PHB is a straight chain homopolymer of D(-)-3-hydroxybutyrate and has the following structural configuration (Figure 1.1):



...(1.1)

The number of monomeric units, n, is largely dependent upon the extraction procedure adopted during the isolation of PHB from cellular material. Neutral solvents are generally preferred for this process (Baptiste, 1962; Alper, 1963; Lundgren et al., 1965) and typically produce n values in the range of 600 - 2500, representing a molecular weight of 60,000 and 250,000, respectively (Lundgren et al., 1965). PHB extracted with hypochlorite, for example, results in low molecular weight preparations of PHB, and values of 5,000 to 15,000 are characteristic of this process (Okamura, 1967). Baptiste (1962) also highlighted the degradative role of trace quantities of water or alcohol during the extraction procedure, since both promote depolymerisation through ester interchange.

The literature is ambiguous with regard to the range of PHB molecular weights that have been found throughout the wide range of bacterial species studied. Although the method of preparation of the polymer significantly modifies the polymeric molecular weight, the extent to which the variation is genetically controlled or

environmentally manipulated is unclear. PHB molecular weight has been shown to vary during the progress of a fermentation (Best, personal communication) and one report suggests that the average molecular weight of the PHB polymer from Azotobacter chroococcum Beij can be marginally varied by the inclusion of phenylacetic acid in the growth medium (Nuti et al., 1972).

The variation in melting point determinations of purified PHB from a wide variety of sources might also reflect differences in polymer chain length. Low molecular weight samples contain a relatively large fraction of chain ends which are thought to lower polymer melting points by acting as impurities (Mandelkern, 1956). The lowest melting points reported (114°C and 120°C) were those obtained from PHB polymer extracted from Bacillus species (Lemoigne, 1927 and 1946). These contrast with the highest melting point value of 188°C reported for PHB extracted from Chromatium (Schlegel & Gottschalk, 1962). In general, however, a large number of determinations have fallen within the range of 168°C - 175°C.

Purified PHB, isolated from a wide range of microbial sources (Rouf & Stokes, 1962; Lundgren et al., 1965; Stockdale et al., 1968), exhibit identical infra-red spectral patterns. The major absorption peak is 5.7 μ, corresponding to the ester carbonyl stretching mode. Low molecular weight samples also exhibit a distinct -OH stretching absorption peak at 2.9 μ due to the presence of a larger proportion of unesterified alcohol groups.

Similar wide ranging X-ray diffraction analysis of purified PHB

(Alper et al., 1963; Lundgren et al., 1965) confirmed crystal structure uniformity in each case. Interestingly, X-ray examination of dried, intact cells yielded identical crystalline patterns, suggesting that native PHB granules are crystalline in the cell and that this crystalline phase is clearly characteristic of the in vivo state of the native polymer.

Conformational studies on the polymer have suggested that it exists in a regular right hand helical conformation (Alper, 1963) with a two fold screw axis along the chain (Okamura & Marchessault, 1967; Cornibert & Marchessault, 1972).

1.2.2 NATIVE PHB GRANULES

In vivo PHB forms a series of discrete granules (diameter 0.24 - 0.7 μm (Ellar et al., 1968)) enclosed by a single membrane which is morphologically distinct from the cytoplasmic membrane (Lundgren et al., 1964; Pfister & Lundgren, 1964; Boatman, 1964). Measurements of membrane thickness from different organisms fall in the range 2.5 - 8 nm (Boatman & Douglas, 1963; Pfister et al., 1964; Lundgren et al., 1964; Wang & Lundgren, 1969). Each granule is composed of fibrils, 100 to 150 angstroms in length, and it is estimated that there are several thousand PHB molecules in each granule (Ellar et al., 1968). This unique morphology has developed, it is postulated, because of simultaneous synthesis and crystallisation at some stage during granule formation (Ellar et al., 1968).

The native granules from B. megaterium were composed of PHB (97.7 %), protein (1.87 %) and lipid (one acetone soluble and one identified as phosphatidic acid) (Griebel et al., 1968). Careful preparation of the granules was required for the expression of in vitro polymerase (synthetase) activity, presumably for the preservation of an intact membrane system. The membrane is also intimately associated with depolymerase activity (Griebel & Merrick, 1971), which was more labile than synthetase activity. This fact, correlated with the different sensitivity of these activities to polypeptide antibiotics, which are known to disrupt membrane organisation (Merrick, 1965), suggests that two separate proteins are responsible for these two activities. The importance of the preservation of an intact membrane to maintain the enzymological functions of polymerisation and depolymerisation have been stressed in several papers (Merrick & Doudoroff, 1964; Merrick et al., 1965; Griebel et al., 1968). However, the molecular architecture of the granule membrane and the precise association of the protein components with the polymer chains has not been elucidated.

1.3 THE PHYSIOLOGICAL SIGNIFICANCE OF PHB

The role of PHB as a carbon storage material has been the subject of much debate during early studies. Lemoigne (1927) described PHB as a reserve material, but after further work (Lemoigne et al., 1950) felt unable to decide whether it represented a waste or storage product. Knaysi (1947) adhered to the concept that the granules were lipoprotein particles arising from the cytoplasmic membrane, representing an abortive attempt at binary fission.

Metabolic studies in washed cell suspensions of B. megaterium (Macrae & Wilkinson, 1958a) established that PHB production was not a mechanism for the removal of toxic acidic products by a process of neutralisation. Accumulation and degradation of polymer had well defined pH optima at 7.5.

Tinelli (1955a & 1955b) discovered that PHB was metabolised during sporulation and deduced that the two processes were intimately associated. Slepecky and Law (1961) expanded these observations during work with B. megaterium and concluded that, while PHB accumulation was not pre-requisite for spore formation, it can be utilised to provide carbon and energy for the sporulation process. Similar observations and conclusions were drawn from studies on Bacillus cereus (Nakata, 1966; Kominek & Halvorsan, 1965) and Clostridium botulinum (Emeruwa & Hawirko, 1973). Recently PHB has been implicated as the principal carbon and energy source utilised by Nocardia asteroides during morphogenesis (Emeruwa, 1981).

A relationship between PHB content and the extent of cyst formation was observed by Stevenson and Socolofsky (1966). They noted that substrates promoting greatest polymer accumulation in Azotobacter vinelandii supported greatest encystment, and when polymer accumulation was reduced, by changes in the carbon or nitrogen supply, the degree of encystment was similarly reduced. Later studies on the same organism (Lin & Sadoff, 1968) revealed that crotonate and D(-)-3-hydroxybutyrate gave increased encystment and increased PHB accumulation, whereas butyrate and butyraldehyde were without effect. The significance of this result was not, however, discussed.

PHB synthesis has also been shown to confer a survival advantage on organisms living in carbon-limited environments. Macrae and Wilkinson (1958a) observed that PHB delayed the autolysis and death of B. megaterium. Stokes and Parson (1968) have shown that cells of Sphaerotilus discophorus, which contained large intracellular stores of PHB, survived longer on starvation in phosphate buffer suspensions than did cells with little or no PHB. Similar results were obtained by Sierra and Gibbons (1962) with Micrococcus halodenitrificans, by Hippe (1967) with a strain of Hydrogenomonas and by Sobek et al. (1966) with Azotobacter agilis. PHB also played a significant role in the survival of Pseudomonas V-19, which can also accumulate glycogen and lipid as its carbon reserve (Zevenhuizen & Ebbinck, 1974).

A theory based on the role of intracellular lipids in bacterial flocculation was proposed by Crabtree et al. (1966). In this theory the assumption was made that, during the process of flocculation, PHB deposits were released to form polymeric bridges between the cells. Shadow cast preparations of well flocculated cells of Pseudomonas V-19 indeed revealed extracellular material between the cells in the form of parallel bundles of straight threads (Deinema & Zevenhuizen, 1971). Furthermore, a good correlation between endogenous lipidaceous reserves and flocculation was also noted during further studies on this organism by Zevenhuizen and Ebbink (1974).

1.4 BIOTECHNOLOGICAL APPLICATIONS OF PHB

Although PHB has been known as a bacterial carbon storage compound for sixty years, it is only within the last decade that the broad spectrum of its potential applications has been fully investigated. To date, most attention has focussed on its properties as one of a family of natural thermoplastic polymers (Holmes, 1985, 1986).

PHB shares a number of analogous properties to several conventional thermoplastics now in use. These include polypropylene and polyethylene tetraphthalate (King, 1982). Its exploitation as a large tonnage plastic has, however, been restricted by production costs, which are presently higher than that of polypropylene. Nevertheless, further oil price increases coupled to improved process technology, may make the large scale production of PHB a viable proposition (Howells, 1982).

Naturally occurring PHB has limited solubility in standard lacquer solvents and is very brittle, giving opaque coatings without any gloss. However, PHB can be chemically modified to form products which may be processed into transparent, glossy, elastic, and hard coatings which are suitable for lacquers (Dhein et al., 1982). PHB can be shaped by extrusion or molding injection and can serve as a substrate for modification to produce coatings . with desirable properties. Desirable polyesters are produced by co-condensing PHB to polyols for making solvent cast articles such as coatings, films or fibres (Dhein et al., 1982).

Another special property which PHB exhibits, and which has already resulted in more immediate small scale use, is that of its biodegradability. Applications include the production of medical disposables, environmentally "friendly " packaging materials and in the encapsulation of agrochemicals (Holmes, 1983). Furthermore, PHB is biocompatable and degraded in living tissue. This property has been exploited in the production of surgical pins and sutures. Further medical applications include its use as an encapsulating material for long term medication dosage (Howells, 1982).

Recently, several groups have investigated the copolymerision of PHB with 3-hydroxyvalerate (HV) during controlled bacterial fermentation (Holmes, 1985; Bloembergen et al., 1986; Doi et al., 1986 and 1988). An increase in HV content of the copolymer leads to a material with lower crystallinity, lower melting point, reduced modulus and greatly enhanced toughness compared to the homopolymer (Webb et al., 1986). This has improved the range of properties and hence of applications for this material.

To date, more speculative ideas for the exploitation of PHB include its use as a source of optically active molecules (Howells, 1982). Furthermore, its piezoelectric properties have stimulated much interest within the electronics industry (Fukada & Ando, 1986).

1.5 POLY-BETA-HYDROXYBUTYRATE METABOLISM

1.5.1 PHB ACCUMULATION DURING BATCH GROWTH

Lemoigne et al. (1950) were the first to observe a relationship between the composition of the growth medium and the quantity of PHB accumulated during batch growth studies on B. megaterium. Macrae and Wilkinson (1958b) extended these observations in an asporogenous strain of the same organism by noting the importance of the C/N ratio in determining the quantity of PHB synthesised. Nitrogen-limited cultures accumulated four times the quantity of PHB produced by cells grown under glucose limitation.

PHB synthesis in A. eutrophus H16 is initiated by one of a number of limitations on growth. Nitrogen-limited cultures can accumulate up to 65% of their dry weight as PHB (Schlegel et al., 1961) and up to 28% during oxygen limitation (Schuster & Schlegel, 1967; Morinaga et al., 1978); phosphate, sulphate, magnesium or potassium also induce PHB synthesis in this organism (Repaske & Repaske, 1976). A study on the formal kinetics of PHB production during batch growth of A. eutrophus H16 has revealed that PHB synthesis occurs in three distinct phases (Sonnleitner et al., 1979): a phase of exponential cell growth and directly associated PHB production; a second transient phase where the rate of PHB production accelerated to a maximum and the rate of rest biomass production approached zero, and a third phase characterised exclusively by PHB synthesis. These data have been used to develop kinetic models to

describe PHB synthesis in this organism (Heinzle & Lafferty, 1980).

The production of high cell mass concentrations with high PHB content has recently been studied in fed-batch culture of Pseudomonas K on methanol (Suzuki et al. 1986a; 1986b; 1986c). PHB synthesis in this organism was induced by nitrogen limitation or by omission of either sulphate, magnesium, iron or manganese from the growth medium. By maintaining a low level of methanol it was possible to to achieve a cell concentration of 206 $gm.l^{-1}$, of which 66 % (136 $gm.l^{-1}$) was PHB. Feeding a small quantity of ammonia resulted in a more rapid increase in the accumulation of intracellular PHB (Suzuki et al., 1986b). Excessive ammonia addition, however, resulted not only in the degradation of accumulated PHB but also in lower PHB synthetic activity. To maximise productivity and concentration the C/N ratio should be controlled according to the increase in PHB content. This resulted in the accumulation of levels of PHB of 136 $gm.l^{-1}$ in 121 hours, as compared to 175 hours (Suzuki et al., 1986c).

Reports of PHB accumulation in methanotrophs have been sporadic with few details on the nature of the nutrient(s) limitation responsible for polymer metabolism. Methanomonas methanica was shown to accumulate up to 25 % of its dry weight as PHB (Harrington & Kallio, 1960; Kallio & Harrington, 1960). PHB has also been identified in many of the methane-utilising strains described by Whittenbury (Whittenbury et al., 1970a; 1970b), Methylovibrio soehngenii (Hazeu & Steenis, 1970), a number of thermophilic methanotrophs (Malashenko, 1976) and in M. trichosporium OB3b (Weaver et al., 1975; Best, 1982). Batch growth studies on four methanotrophs suggested that oxygen

limitation was responsible for PHB synthesis (Tezuka et al., 1980). One organism, Methanomonas margaritae, accumulated 20 % of its dry weight as PHB under these conditions.

1.5.2 CHEMOSTAT GROWTH STUDIES

The limitations of batch growth studies for precise identification of the nutrient responsible for the synthesis/mobilisation of PHB are apparent. Batch grown cells are subject to variation in nutrient levels with time and, furthermore, undergo physiological changes during the onset of the stationary phase at a time when the deposition of PHB, in most organisms, is maximal. Subsequently, the problem of differentiating between an apparent nutrient limitation and a real nutrient limitation would be difficult, if not impossible, to solve without recourse to chemostat growth analysis.

Wilkinson and Munro (1967) appear to have been the first to evaluate the factors responsible for the production of PHB during chemostat growth. They found that B. megaterium KM, grown with either nitrogen, sulphur, potassium as the limiting factor, accumulated PHB with a maximum level at a dilution rate of 0.4 h^{-1}. The somewhat inexplicable observation that carbon limited cultures also accumulated appreciable quantities of PHB (12 % of the dry weight), possibly suggests that PHB biosynthesis in this organism is not a metabolic "shunt" for the disposal of carbon in excess of cellular requirements. The strain of B. megaterium used by Wilkinson and Munroe (1967) had, however, undergone several significant changes since its

original isolation, having lost the ability to form spores and having acquired the ability to grow in simple synthetic medium and under conditions of continuous culture. The results should therefore be treated with some reserve.

The difficulties experienced by Senior et al. (1972) in determining the nature of the nutrient limitation responsible for the synthesis of PHB in batch grown cultures of A. beijerinkii was resolved by chemostat studies. The studies indicated: (i) nitrogen-limited cultures did not accumulate PHB over a wide range of growth rates (D = 0.052 - 0.205 h^{-1}). (ii) PHB (3 % dry weight) was produced in glucose-limited cultures at low dilution rates. (iii) Oxygen-limited cultures produced large quantities of PHB which ranged from 20 % dry weight at the highest specific growth rate (D = 0.252 h^{-1}) to 45 % at the lowest growth rate (D = 0.049 h^{-1}). The relaxation of oxygen-limitation resulted in an immediate decrease in the PHB content of the organism. (Senior et al., 1972; Jackson & Dawes, 1976). At low dissolved oxygen tension, PHB accumulation in ammonium-grown organisms differed from those fixing atmospheric nitrogen (Ward et al., 1977). When the culture was taken through a series of steady states of increasing oxygen tension, the PHB content decreased regularly from its maximum value of 70 %, at the lowest level, to a minimum of 5 % of the dry weight at an oxygen tension of 1.25 %. By increasing this value to 2 %, the PHB content of the cells rose to 35 % of the dry weight. During this time the culture was thought to pass from an oxygen limitation to a nitrogen limitation; both sets of nutrient limitation were apparently responsible for initiating PHB synthesis. Ward et al. (1977) rationalised the different behaviour

observed between nitrogen-fixing and ammonium grown A. beijerinkii in terms of the greater demand for ATP and reducing power during nitrogen fixation.

1.6 THE ENZYMOLOGY OF PHB METABOLISM

1.6.1 INTRODUCTION

The first step in the synthesis of PHB involves the condensation of two molecules of acetyl-CoA by beta-ketothiolase to form acetoacetyl-CoA and free CoASH. The reduction of acetoacetyl-CoA and the subsequent polymerisation of D-(-)-3-hydroxybutyryl-CoA results in the formation of the product of the pathway. PHB mobilisation occurs via the corresponding free acids, D(-)-3-hydroxybutyrate and acetoacetate. CoASH transfer to acetoacetate regenerates acetyl-CoA, thus completing the cyclical pathway (Figure 1.1).

This section summarises the role of each enzyme in the pathway of polymer metabolism in terms of its kinetic and regulatory properties where known. A detailed discussion on the properties of the soluble enzymes in the pathway, D(-)-3-hydroxybutyrate dehydrogenase (Section 3.1), acetoacetyl-CoA synthetase/acetoacetate:succinyl-CoA CoA transferase (Section 4.1), beta-ketothiolase (Section 5.1) and acetoacetyl-CoA reductase (Section 6.1) are provided elsewhere and are not covered in this section.

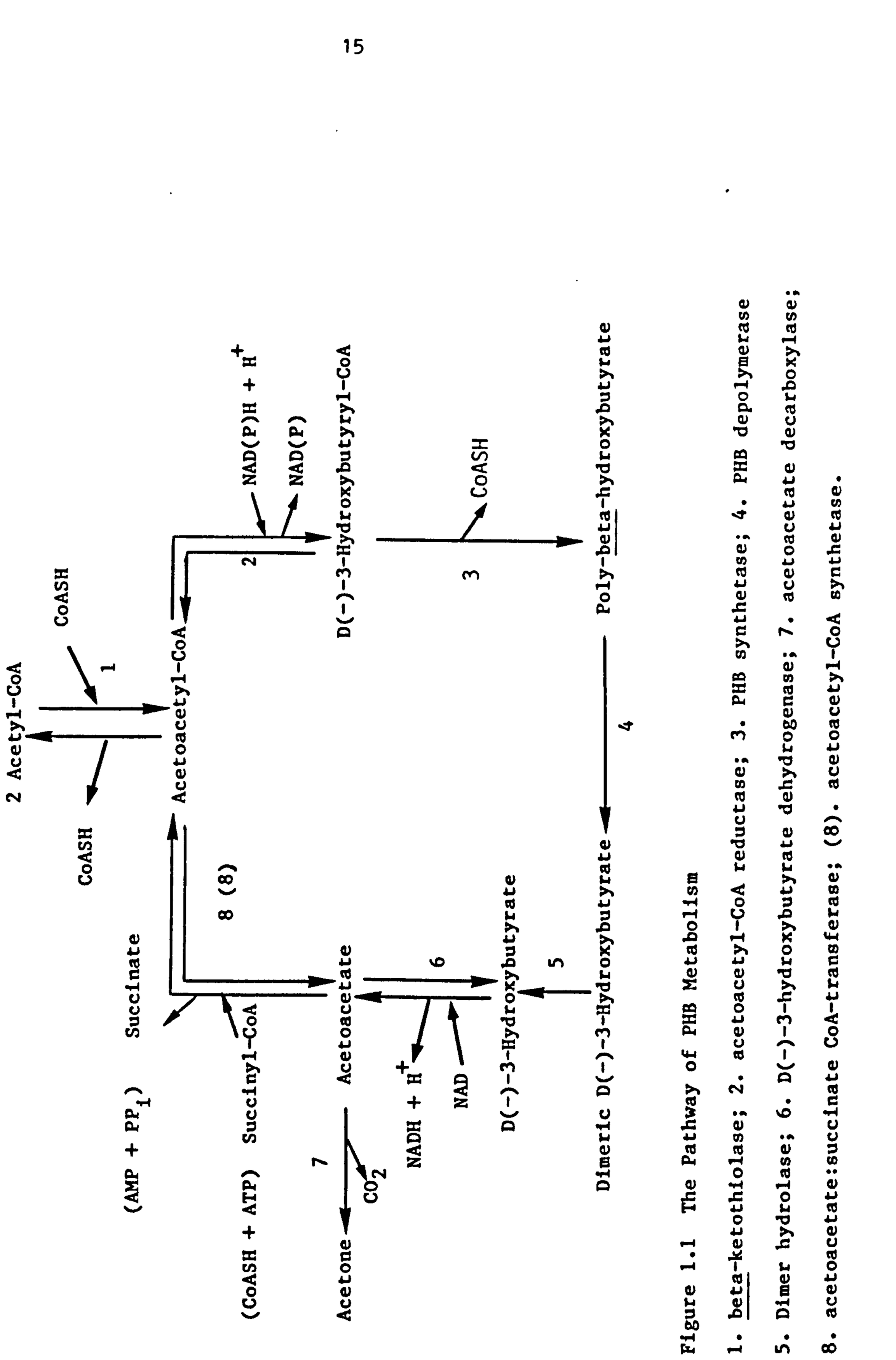


Figure 1.1 The Pathway of PHB Metabolism

1. beta-ketothiolase; 2. acetoacetyl-CoA reductase; 3. PHB synthetase; 4. PHB depolymerase
5. Dimer hydrolase; 7. acetoacetate decarboxylase; 6. D(-)-3-hydroxybutyrate dehydrogenase;
8. acetoacetate:succinate CoA-transferase; (8). acetoacetyl-CoA synthetase.

1.6.2 THE ENZYMOLOGY OF PHB SYNTHESIS

1.6.2.1 PHB SYNTHETASE

The polymerisation of D(-)-3-hydroxybutyryl-CoA to form PHB, with the subsequent liberation of free CoASH, was first demonstrated in cell free extracts of B. megaterium and Rhodospirillum rubrum (Merrick & Doudoroff, 1961). In both bacteria, PHB synthetase activity was associated with the native PHB granules isolated by low speed centrifugation of the respective cell free extracts.

Later studies (Merrick & Doudoroff, 1964; Merrick 1965) using B. megaterium revealed that synthetase activity was closely associated with the granule membrane. Purification of the granules lead to a dramatic loss of activity (90 %) but an apparent K_m of 9.25×10^{-5} M for D(-)-3-hydroxybutyryl-CoA was determined (Griebel et al., 1968). It was postulated that initial granule formation was promoted by the aggregation of the synthetase enzyme into a micellar form, subsequent polymer synthesis occurring within the protein coat and giving rise to the polymer fibrils parallel to each other (Griebel et al., 1968). In a later study on the same system (Griebel & Merrick, 1971) mild alkaline hydrolysis was used to solubilise protein from the native PHB granules. Both the extracted granules and the solubilised protein were devoid of synthetase activity, but this could be partially restored by combination of the two (5-6 % of original activity). Further examination of the solubilised protein identified a high molecular component, which possessed synthetase activity in combination with the extracted granules. The active component was extremely labile,

although some stability was afforded by manganese chloride (1 mM), glycerol (20 % w/v) and 2-mercaptoethanol (5 mM) in combination. Preincubation was required for the reassociation of the protein with the extracted granule and the K_m for this 'solubilised' synthetase was 3.12×10^{-4} M, the lower value possibly reflecting an altered conformation of the protein/polymer interaction in the reconstituted system. A two stage reaction mechanism was proposed (equation 1.1) with an acyl-enzyme intermediate complex but the characteristics of the protein deemed responsible for synthetase activity were not further elucidated.

(1) D(-)-3-Hydroxybutyryl-CoA + Synthetase-SH ⟶

3-Hydroxybutyryl-S-Synthetase + CoASH

(2) 3-Hydroxybutyryl-S-Synthetase + PHB primer (n units) ⟶

PHB primer (n + 1 units) + Synthetase-SH ...(1.1)

PHB synthetase activity in Zoogloea ramigera I-16-M has been found in two forms, one particulate and one membrane bound (Fukui et al., 1976; Tomita et al., 1983). It has been inferred that both activities are due to the same protein but the location of this protein is dependant upon the prevailing growth conditions. The enzyme is exclusively soluble in glucose-limited cells, devoid of PHB, and particulate (granule bound) during polymer synthesis. A transition between the particulate and the soluble forms was demonstrated during the starvation of PHB rich cells. Both forms have been partially purified, but extreme lability was noted as purification proceeded. Attempts to solubilise the particulate PHB synthetase by alkaline hydrolysis or by mild detergents resulted in a complete loss of enzyme

activity. The particulate enzyme did not display conventional Michaelis-Menten kinetics, and an apparent K_m of 2.1 x 10^{-4} M was estimated for D(-)-3-hydroxybutyryl-CoA. A Hill coefficient of 1.3 suggested some degree of positive cooperativity and at least two binding sites for D(-)-3-hydroxybutyryl-CoA. The soluble form of the enzyme displayed linear Michaelis-Menten kinetics with a K_m of 5.3 x 10^{-5} M. The difference in kinetic constants between the two enzymes for D(-)-3-hydroxybutyryl-CoA may reflect conformational changes in the enzyme on binding to the PHB granule.

1.6.3 THE ENZYMOLOGY OF PHB DEGRADATION

1.6.3.1 DEGRADATION OF EXTRACELLULAR PHB

A number of bacteria capable of utilising purified PHB as a sole source of carbon and energy were isolated and partially characterised by Delafield et al. (1965b). In each case PHB was hydrolysed by an extracellular depolymerase to a mixture of its monomeric and dimeric constituent units. The enzyme was produced constitutively during growth on a wide range of carbon sources.

Previously, Chowdhury (1963) isolated two Pseudomonads which produced extracellular PHB depolymerase. Further investigation of one strain, Pseudomonas PL, led to the suggestion that depolymerase activity was induced by PHB granules. However, in view of the insolubility of PHB, enzyme induction was more likely to be initiated by the soluble esters of D(-)-3-hydroxybutyrate, produced by constitutive, non-specific esterase activity (Delafield et al.,

1965b). The enzyme excreted by this organism had a high pH optimum of 9.8 and a broad substrate specificity, hydrolysing the following (in descending order of activity) beta-hydroxybutyric acid propyl ester >PHB > ethyl acetate > tributyrin > para-nitrophenyl acetate >para-nitrophenyl caprylate.

Lusty and Doudoroff (1966) purified two PHB depolymerases from P. lemoignei to electrophoretic homogeneity. The enzymes, designated A and B, had molecular weights of 38,700 daltons and 37,500 daltons respectively and displayed similar physical properties, substrate specificity and susceptability to chelating agents. They were nevertheless distinct with respect to the course of PHB digestion: with A, little trimer accumulated during PHB hydrolysis and the final monomer/dimer ratio was less than 0.2. Trimer accumulated as the principal end product of B until the polymer had completely disappeared, after which time, trimer hydrolysis resulted in a final monomer/dimer ratio of 0.75. Further differences in the two enzymes were noted in their response to monovalent and divalent cations and in their immunological properties.

Unlike the extracellular depolymerase from P. lemoignei the equivalent enzyme from Alcaligenes faecalis (Tanio et al., 1982) was not constitutive; the enzyme was induced in a medium containing PHB. Although depolymerase activity was highest on purified PHB (K_m = 0.78 x 10^{-6} M), trimer (K_m = 4.5 x 10^{-6} M), tetramer and pentamer were also hydrolysed by this enzyme. Studies on the polarity of substrate hydrolysis indicated that the enzyme attacked the free -OH terminus of PHB, releasing dimers. Further, hydrolysis of the dimer was effected

by an oligomer hydrolase (Shirakura et al., 1983) prior to transport of the monomer into the cell.

1.6.3.2 PHB DEPOLYMERASE

PHB depolymerase catalyses the initial hydrolysis of native PHB with the subsequent liberation of low molecular weight oligomers. The enzyme has been isolated and characterised from the soluble cell extracts of several bacteria, which include R. rubrum (Merrick & Doudoroff, 1964), B. megaterium (Gavard et al., 1966) and A. eutrophus H16 (Hippe & Schlegel, 1967).

Early studies showed that the PHB granules from R. rubrum possessed both synthetase and depolymerase activities and that these granules rapidly autohydrolysed (Merrick & Doudoroff, 1961 and 1964). In contrast, the PHB granules from B. megaterium only possessed synthetase activity (Griebel et al., 1968) and did not undergo appreciable self-digestion. Studies on the PHB depolymerase used a mixed system, comprised of soluble extracts of R. rubrum, prepared from PHB depleted cells, and the native granules prepared from B. megaterium (Merrick & Doudoroff, 1964). The results suggested that the depolymerising enzyme system is dependent upon the interaction of a thermostable activator and a heat labile depolymerase (Merrick & Doudoroff, 1964). Their successive action resulted in the breakdown of PHB to a mixture of D-(-)-3-hydroxybutyrate (80 - 85 %) and a small amount of soluble dimeric ester (15 - 20 %) (Merrick & Yu, 1966). The soluble enzyme system, however, would not attack native PHB granules isolated from B. megaterium that had been treated with various

physical or chemical agents, nor would it attack chemically purified polymer (Merrick & Doudoroff, 1964). This suggests that a labile component, which appears to be associated with the membraneous coat surrounding the granule (Lundgren et al., 1964; Merrick, 1965; Merrick et al., 1965), is also necessary for depolymerisation to proceed. Microscopic examination of granules inactivated in this way displayed morphological changes, which were characterised by membrane fragmentation, loss of coalescence and surface irregularities (Merrick et al., 1965). Furthermore, antibiotics known to disrupt membrane integrity also inhibit depolymerisation (Merrick, 1965).

Later, the labile particulate component was removed from the native PHB granules of B. megaterium (Griebel & Merrick, 1971) by mild alkali extraction. The extracted granules no longer required pretreatment with the activator but were susceptible to direct hydrolysis by the depolymerase. The solubilised protein, however, when added back to the system, prevented polymer hydrolysis. This inhibition can be reversed by the activator.

This study suggests that the mechanism for the control of PHB mobilisation may parallel enzyme induction in bacteria. A small activator protein bound to the granule may prevent the binding or activity of the PHB depolymerase during accumulation of the polymer. Degradation of the polymer may be initiated through the production of an activator which interacts with the inhibitor protein, permitting hydrolysis to begin.

1.6.3.3 D(-)-3-HYDROXYBUTYRATE DIMER HYDROLASE

The dimer hydrolases described to date are characterised by their narrow range of substrate specificities for the oligomers of D(-)-3-hydroxybutyrate. The oligomeric chain length hydrolysed varies between species, but falls within the range 2-5 monomeric units. The hydrolysis of longer chain lengths has not been investigated, although high molecular weight polymer is not a substrate.

During early studies, Merrick and Doudoroff (1964) demonstrated esterase activity in crude extracts of R. rubrum. The enzyme was capable of hydrolysing the dimeric ester of 3-hydroxybutyric acid, to its monomeric units.

The purification of dimer hydrolase from P. lemoignei was described by Delafield et al. (1965a and 1965b). The enzyme was unusually specific in comparison with other bacterial lipases, displaying principal specificity for the CH_3-CHOR-CH_2-COOR´ portion of the substrate, which must possess the D(-) configuration. The terminal carbon may be either a free carboxyl group (as in the dimer) or esterified (as in the bromophenylacyl ester of the dimer). The D-oxybutanoate moiety could not be substituted by L-oxybutanoate, DL-oxypropionate, or n- or sec-oxybutane. The enzyme was less specific for the D-3-hydroxybutanoyl (R) moeity of the dimer, since this could be substituted by the L-isomer or a butanoyl group. The trimeric ester of D-oxybutanoate was cleaved, albeit slowly.

The dimer hydrolase from R. rubrum differed markedly from the

Pseudomonas enzyme in both substrate stereospecificity and chain length of oligomer hydrolysed (Merrick & Yu, 1966). The DL dimer was susceptible to attack, and the trimeric ester was hydrolysed at a higher rate than the DD-dimer. This suggests that the trimer may be the physiological substrate for this enzyme in vivo. Trimeric esterase activity was similarly noted in B. megaterium (Gavard et al., 1966) together with pentameric esterase activity. However, it was thought that its normal physiological function involved dimer hydrolysis.

An investigation into the stereospecificity and extent of oligomer chain length hydrolysis was performed with the electrophoretically homogeneous dimer hydrolase from Z. ramigera I-16-M (Tanaka et al., 1981). The enzyme hydrolyses the trimeric, tetrameric and pentameric esters of D(-)-3-hydroxybutyrate at higher rates than the dimeric ester. Examination of product release during hydrolysis of the methyl ester of DDD- trimer indicated a sequential release of monomer from the terminus with a free 3-hydroxyl group.

Clearly in view of the wide difference in chain length specificities of the hydrolases examined to date the classification of this enzyme as an oligomeric hydrolase might be more appropriate.

1.7 METHANOTROPHS: AN INTRODUCTION

Methanotrophs are bacteria that use methane as the sole source of carbon and energy and are members of the group of organisms known as the methylotrophs. These are defined as organisms which grow on reduced carbon compounds containing one or more carbon atoms but no carbon-carbon bonds (Anthony, 1982).

Although over one hundred different strains of methanotrophs have been isolated and characterised, a formal taxonomic system has yet to be detailed. Most of these strains were isolated by Whittenbury and his colleagues (1970b), who classified the isolates into Type I (Methylococcus, Methylomonas and Methylobacter) and Type II (Methylosinus and Methylocystis) based upon the intracytoplasmic membrane arrangement. This classification was substantiated with the discovery of a concomitant difference in the nature of the carbon asssimilation pathway utilised (Lawrence & Quayle, 1970) and the presence or absence of a complete tricarboxylic acid cycle (Davey et al., 1972). Other basic characteristics which are used in the taxonomy of these organisms include gross morphology (e.g. rods, cocci, vibrios), structural features (e.g. spore formation, rosette formation, flagellae) and nitrogen fixation etc. (for a review see Anthony, 1982).

Recent studies have indicated that lipopolysaccharides, phospholipids and hydroxy fatty acids act as potential 'signatures'

for a range of methylotrophic bacteria, including some methanotrophs (Nichols et al., 1985). Furthermore, Meyer and co-workers (1986) have indicated that GC ratios (guanine:cytosine) can be directly correlated to the 'Type' classification of Whittenbury et al. (1970b).

More complete taxonomic systems have been proposed (Romanovskaya, 1978; Romanovskaya et al., 1978; Patt et., 1974; Patel et al., 1978) to include new obligate strains and the more recently isolated facultative methanotrophs. Claims for the isolation of true faculative methanotrophs, however, should be treated with caution in the light of the discovery that Methylobacterium ethanolicum has since been found to be a syntrophic association of a true methanotroph (Methylocystis sp. POC) and a facultative methylotroph (Xanthobacter sp. H4-14) which were not separable under the usual conditions of growth of these organisms (Lidstrom-O'Connor et al., 1983).

Recently, a third group of methanotrophic bacteria has been recognised, Type X, the sole representative being Methylococcus capsulatus (Bath) (Whittenbury & Dalton, 1981). Whilst the major carbon assimilation route in this organism is clearly via the RMP pathway, there is evidence for two minor carbon incorporation routes, the Benson-Calvin pathway of autotrophic carbon dioxide fixation and the serine pathway, thus setting this organism apart from Type I or II genera (Whittenbury, 1981).

At present the 'Type' classification of Whittenbury and his colleagues remains both the most popular and straightforward system for the primary classification of methanotrophs.

1.8 OXIDATIVE CARBON METABOLISM

The pathway of methane oxidation to carbon dioxide (Figure 1.2) via methanol, formaldehyde and formate has been well established for many years. Although considerable attention has been focussed on the enzymes of the oxidative sequence, until recently little was known of the mechanisms involved, particularly those associated with methane and methanol oxidation. Significant advances have now been made towards elucidating the mechanism of these steps at the molecular level. These will be discussed in the following section.

1.8.1 METHANE MONOOXYGENASE

The oxidation of methane to methanol in methanotrophic bacteria is catalysed by a multicomponent monooxygenase (Tonge et al., 1977; Colby & Dalton, 1978; Stirling & Dalton, 1979; Patel et al., 1982). Methane monooxygenase or 'MMO' has been purified and extensively studied from several methanotrophs including M. capsulatus (Bath) (Colby & Dalton, 1978; Colby & Dalton, 1979; Dalton 1980; Woodland & Dalton, 1984), M. trichosporium OB3b (Tonge et al., 1975; Tonge et al., 1977) as well as the facultative methanotroph, Methylobacterium sp. strain CRL-26 (Patel, 1984; Patel & Salvas, 1987). One of the outstanding characteristics of this enzyme is its broad substrate specificity, catalysing the hydroxylation of methane in addition to the oxygenation of various hydrocarbons and cyclic, alicyclic and aromatic compounds (Colby & Dalton, 1976; Dalton, 1980a, 1980b; Higgins et al., 1981; Patel et al., 1982).

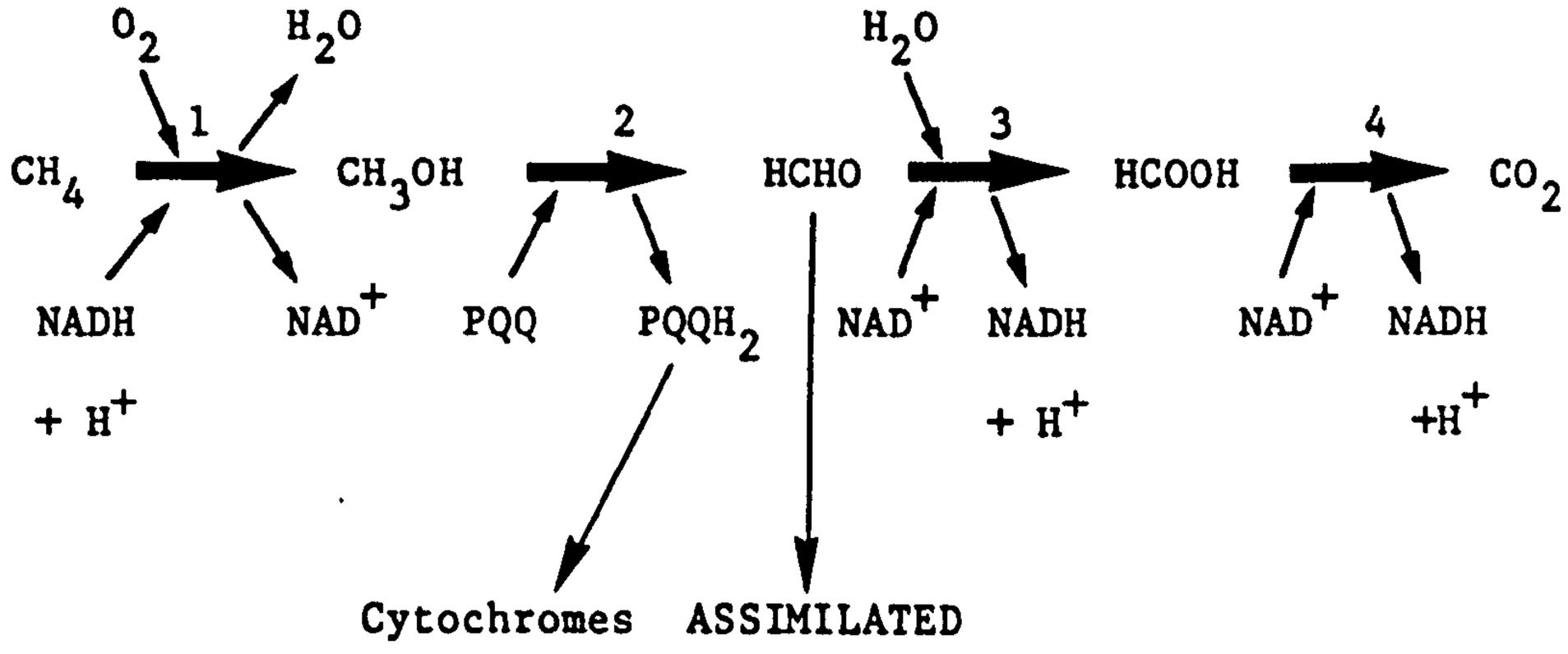


Figure 1.2 Pathway of Carbon Oxidation in Methanotrophs

1. Methane monooxygenase (MMO); 2. Methanol dehydrogenase;

3. Formaldehyde dehydrogenase ; 4. Formate dehydrogenase.

Abbreviations: PQQ, pyrrolo-quinone.

MMO can be expressed in the soluble or particulate fraction of the cell in response to the nutrient status of the growth medium. Stanley et al. (1983) demonstrated that the copper concentration during growth of M. capsulatus (Bath) was highly determinative of the location of the MMO, it being membrane bound at high copper : biomass ratio and soluble under copper stress conditions. The same effect was noted with M. trichosporium OB3b in the same study and later confirmed by Burrows et al. (1984). The precise mechanism(s) by which copper availability regulates the intracellular location of MMO, however, remains to be establised.

MMOs from most sources appears to be obligatorily NAD(P)H linked; reports that ascorbate might serve as electron donor for the particulate enzyme from M. trichosporium OB3b (Tonge et al., 1975) have not been substantiated (Stirling & Dalton, 1979; Patel et al., 1982). Furthermore, the ability of a methanol dehydrogenase couple (Tonge et al., 1975; Tonge et al., 1977) to serve as an electron donor to MMO has not been verified in other organisms (Dalton et al., 1984). Indeed, compounds which when metabolised yield NADH directly (e.g. formaldehyde and formate) in whole cells will act as donors for MMO in vivo (Hou et al., 1980; Stirling & Dalton, 1980).

1.8.2 METHANOL DEHYDROGENASE

Methanol oxidation in methanotrophs is catalysed by a NAD(P) independant methanol dehydrogenase (MDH) (alcohol: (acceptor) oxidoreductase, EC 1.1.99.8) of the type first described by Anthony &

Zatman (1964). MDHs have been characterised from a large number of methylotrophic bacteria (Anthony, 1982) and several methanotrophs (Patel et al., 1972; Patel & Felix, 1976; Wadzinski & Ribbons, 1975) and, in general, appear to be closely similar. A major unifying property of the various MDHs is their breadth of substrate specificity for primary aliphatic alcohols and the dual activity of the enzymes towards primary alcohols and formaldehyde. Oxidative activity towards formaldehyde is probably a consequence of the compound existing in hydrated form in aqueous solution (Sperl et al., 1974). The high pH optima of this enzyme (generally in the range 9.0 - 10.0) for methanol and the requirement for ammonium ions or a primary amine in the presence of an artificial electron acceptor for the expression of in vitro activity are all common properties of this enzyme.

MDH appears to be coupled to the electron transport chain at the level of cytochrome c (Ohta & Tobari, 1981; Beardmore-Gray et al., 1983). Electron transfer from methanol to cytochrome c is mediated via a novel prosthetic group, pyrrolo-quinoline quinone (PQQ). The observation that a quinone derivative was the cofactor for MDH was made as a result of studies conducted on the purified enzyme from Hyphomicrobium X (Duine et al., 1979). Electron spin resonance studies (Westerling et al., 1979) and X-ray crystallographic studies (Salisbury et al., 1979) were used to propose a structure for the prosthetic group. Confirmation of the structure was later presented by Duine and co-workers (1980).

A mechanism for the oxidation of methanol involving the prosthetic group was proposed by Forrest et al. (1980). In this

mechanism (Fig 1.3) PQQ forms an addition complex with the amino group of a lysine residue (or with ammonia or a primary amine) at the C_4 position (II). 1,4 elimination of water produces the quinone analogue (III) in the active enzyme. 1,4 addition of the primary alcohol allows a cyclic rearrangement with release of the oxidised product (formaldehyde).

Recently Duine and co-workers (1984) have reported that M. capsulatus (Bath) possesses NAD-linked MDH activity in addition to the classical MDH. The extent of this occurrence in methanotrophs has not yet been ascertained.

1.8.3 FORMALDEHYDE DEHYDROGENASE

Several different types of formaldehyde dehydrogenase activity have been found in various C-1 utilising bacteria (Anthony, 1975; Stirling & Dalton, 1978). They can be classified into two groups: 1. NAD(P)-linked enzymes, some requiring glutathione for activity, 2. NAD(P)-independent enzymes that require artificial electron acceptors, e.g. phenazine methosulphate (PMS) or dichlorophenolindophenol, for in vitro activity. Both groups encompass enzymes which are either specific for formaldehyde, or non-specific aldehyde dehydrogenases.

NAD-linked formaldehyde dehydrogenase activities have been demonstrated in two strains of M. methanica (Harrington & Kallio, 1960; Johnson & Quayle, 1964), M. organophilum (O'Connor & Hanson, 1977) and M. trichosporium OB3b (Stirling & Dalton, 1979). Stirling and Dalton (1978) purified a NAD(P)-linked formaldehyde dehydrogenase

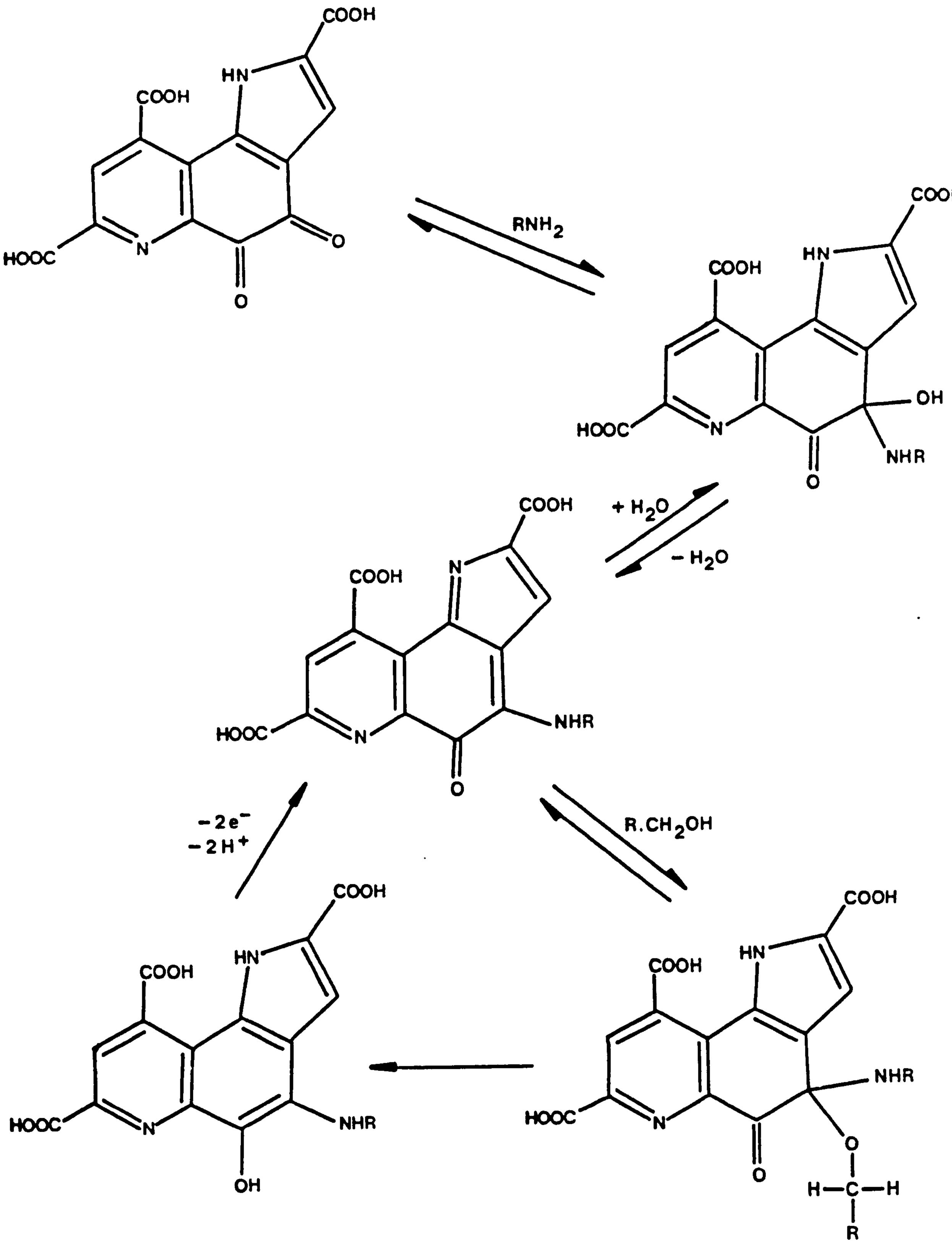


Figure 1.4 Proposed Mechanism of Action of Pyrrolo-quinoline-quinone Oxidation of Primary Alcohols (from Salisbury et al., 1979).

from M. capsultus (Bath) which was capable of oxidising glycollaldehyde, glyoxal and glyceraldehyde. The enzyme was dimeric (subunit M_r 57,000) and required a small, heat sensitive component (peptide or protein) for activity.

NAD-independant formaldehyde dehydrogenases possess, in general, very broad specificities, one exception being the enzyme purified from Pseudomonas RJ1 which is reported to be specific for formaldehyde (Mehta, 1975). They oxidise a wide range of aliphatic and aromatic aldehydes, glyoxal, methylglyoxal, glyoxalate, glyceraldehyde, glycollaldehyde and glutaraldehyde (Johnson & Quayle, 1964; Patel et al., 1979a, 1980; Marison & Attwood, 1980).

The enzymes purified from Methylosinus trichosporium strain PG and Methylomonas methylovora have molecular weights of 43-45,000 and are composed two subunits of molecular weight 23,000. Evidence suggests that both enzymes are haemoproteins with no associated flavin prosthetic groups (Patel et al., 1979a; 1980). Anthony (1982), however, suggested that the haem characteristic might be due to contamination of the enzyme with cytochrome c. The role of this enzyme in C_1 metabolism is uncertain and the work of Marison and Attwood (1980) suggests that the dye-linked enzyme is probably a general aldehyde dehydrogenase, for which formaldehyde is a poor substrate, rather than an enzyme with a major role in the dissimilatory pathway for C_1 compounds.

1.8.4 FORMATE DEHYDROGENASE

Formate dehydrogenase catalyses the oxidation of formate to carbon dioxide and water. The majority of NADH required for methanotrophic cell growth is thought to be generated by this reaction. NAD-dependant activities have been demonstrated in M. methanica (Johnson & Quayle, 1964; Ben-Bassat & Goldberg, 1977), M. capsulatus (Texas) (Patel & Hoare, 1971; Strom et al., 1974; Ribbons & Wadzinski, 1976), M. trichosporium OB3b (Weaver & Duggan, 1975), M. trichosporium species PG (Patel et al., 1979) and Methylococcus mobilis (Hazeu et al., 1980).

1.9 CARBON INCORPORATION IN METHANOTROPHS

1.9.1 INTRODUCTION

The ribulose monophosphate (RMP) pathway and the serine pathway represent the major carbon assimilation routes in Type I and Type II methanotrophs respectively. In both cases carbon is assimilated at the level of formaldehyde; Type II organisms additionally incorporate carbon in the form of CO_2 via the serine pathway. The elucidation, characterisation and evolution of these pathways have been reviewed extensively and the reader is referred to these works for further information (Wolfe & Higgins, 1979; Colby et al., 1979; Higgins et al., 1981; Whittenbury, 1981; Anthony, 1982). Schematic representations of the pathways together with a brief description of their operation is presented here.

1.9.2 THE RIBULOSE MONOPHOSPHATE (RMP) PATHWAY

The RMP pathway of carbon assimilation was first proposed by Kemp and Quayle (1967) when short term incubations of M. methanica with ^{14}C-labelled methanol or formaldehyde resulted in early labelling of sugar phosphates, particularly hexose phosphate. Subsequent work (Lawrence et al., 1970; Kemp, 1974) showed the key reactions of the pathway to be those catalysed by 3-hexulose phosphate synthase and 3-hexulose phosphate isomerase.

The RMP pathway is conveniently divided into three stages (Figure 1.4). The first part of the sequence involves the aldol condensation of formaldehyde with three molecules of hexulose 5-phosphate via hexulose phosphate synthase to produce 3-hexulose 6-phosphate. Isomerisation of hexulose phosphate leads to the formation of fructose 6-phosphate (FMP) (three molecules). In the second part of the sequence FMP is converted to either fructose 1,6-biphosphate (FBP) or to 2-keto 3-deoxy 6-phosphogluconate (KDPG). These molecules are then cleaved by aldolases to glyceraldehyde 3-phosphate plus the 'product' of the pathway, which is either pyruvate (from KDPG) or dihydroxyacetone phosphate (from FBP). Stage 3 involves the regeneration of 3-ribulose 5-phosphate for fixation of further formaldehyde molecules. There are two possible cleavage enzymes and two rearrangment sequences giving four potential variants of the pathway (Figure 1.4). Aspects on the regulation of the RMP pathway have been highlighted elsewhere (Quayle, 1980; Anthony, 1982).

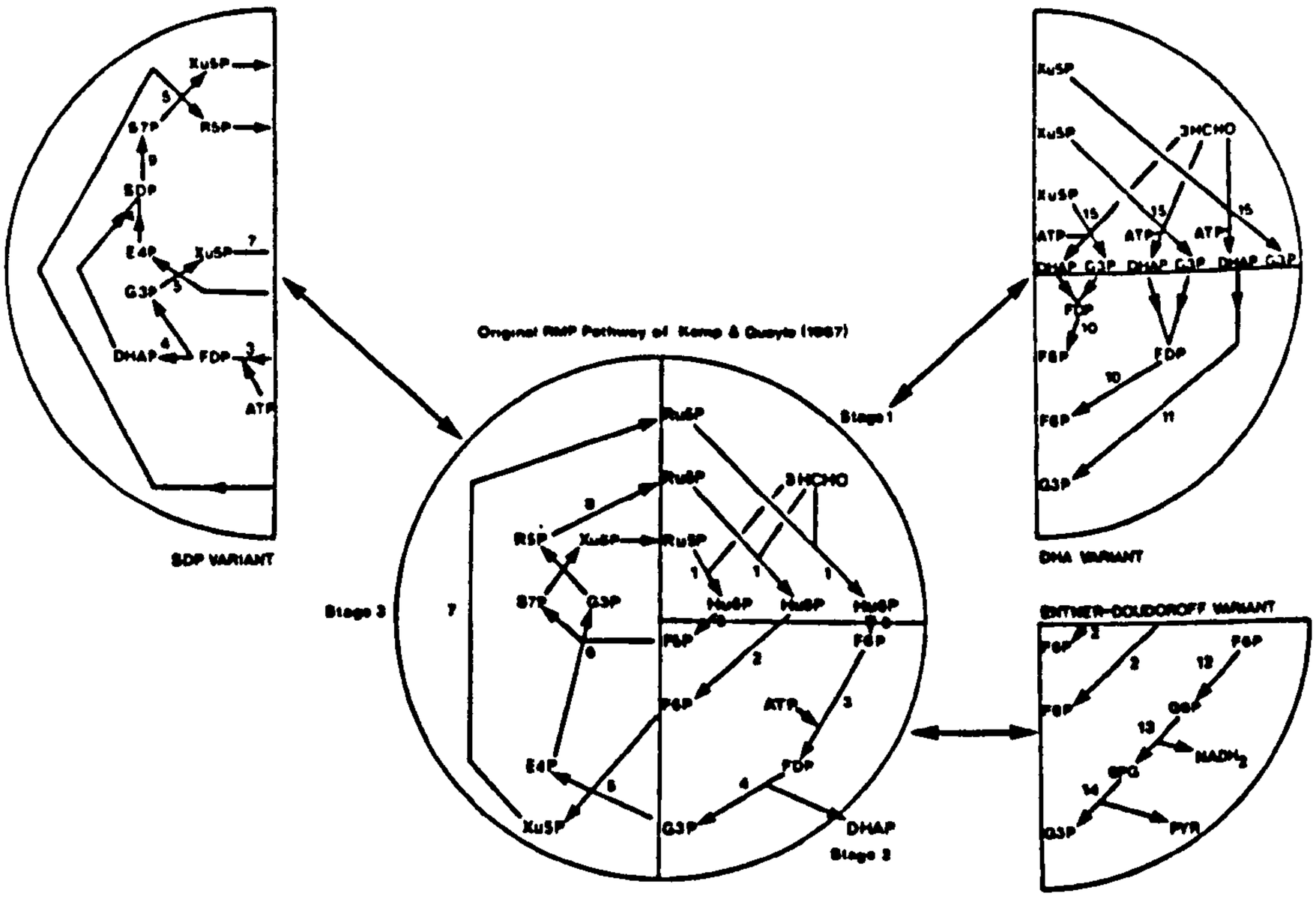


Figure 1.4 The Ribulose Monophosphate (RMP) pathway of Carbon Assimilation

RU5P, Ribulose-5-phosphate; Hu6P,D-erythro-L-glycero-3-hexulose-6-phosphate; F6P, fructose-6-phosphate; FDP, fructose-1,6-diphosphate; G3P, glyceraldehyde 3-phosphate; DHA, dihydroxyacetone; DHAP, DHA-phosphate; E4P, erythrose 4-phosphate; Xu5P, xylulose-5-phosphate; S7P, sedoheptulose-7-phosphate; SDP, sedoheptulose-1,7-diphosphate; R5P, ribulose-5-phosphate; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; PYR, pyruvate.

1. 3-hexulose phosphate synthase; 2. phospho-3-hexuloisomerase; 3. 6-phosphofructokinase; 4. fructosediphosphate aldolase; 5. transketolase; 6. transaldolase; 7. ribulosephosphate epimerase; 8. ribulosephosphate isomerase; 9. sedoheptulose diphosphatase; 10. fructose diphosphatase; 11. triosephosphate isomerase; 12. glucosephosphate isomerase; 13. glucose-6-phosphate dehydrogenase; 14. 6-phosphogluconate dehydratase+phospho-2-keto-3-deoxygluconate aldolase; 15. transketolase+triokinase. (after Colby et al., 1979).

Whilst the major carbon assimilation route in Type I methanotrophs is clearly via the RMP pathway, the presence of enzyme activities associated with the serine pathway (see below) have been reported. Low activity of both hydroxypyruvate reductase and malyl-CoA lyase were detected in M. capsulatus (Texas) and M. methanica (Salem et al., 1973; Strom et al., 1974). Of the twelve Type I isolates examined by Trotsenko (1976), low activities of hydroxypyruvate reductase and serine-glyoxylate aminotransferase were found in eleven cases. Only one was found to assimilate carbon exclusively through the RMP pathway.

In the Type X organism, M. capsulatus (Bath) there is some evidence to suggest that two minor carbon incorporation pathways operate in addition to the RMP pathway (Whittenbury, 1981). Firstly the presence of low activities of two enzymes of the Benson-Calvin pathway of autotrophic carbon dioxide fixation, phosphoribulokinase and ribulose 1,5 bisphosphate carboxylase would enable the conversion of ribulose 5-phosphate to ribulose 1,5 bisphosphate and incorporation of CO_2, ultimately yielding 3-phosphoglycerate for assimilation. Secondly, the early distribution of about 90 % of ^{14}C-formate assimilated was found to be incorporated into serine and glycine. This fact, and the presence of hydroxypyruvate reductase, may indicate that M. capsulatus (Bath) can assimilate some carbon via a serine pathway.

Although the presence of alternative carbon assimilatory pathways may not indicate the operation of a complete cycle, it has been suggested (Colby, 1979) that under certain environmental conditions their operation might create a distinct survival advantage.

1.9.3 THE SERINE PATHWAY

Type II methanotrophs, including M. trichosporium OB3b, assimilate formaldehyde via the the serine pathway (Lawrence et al., 1970; Lawrence & Quayle, 1970). The serine pathway was first proposed by Large et al. (1961) as a result of ^{14}C-labelling studies in facultative methylotrophs. Key enzyme activities of the pathway include hydroxypyruvate reductase, serine transhydroxymethylase and malyl-CoA lyase. In many instances however, detection of high levels of hydroxypyruvate reductase alone have been used to indicate the operation of the pathway (Lawrence et. al., 1970; Lawrence & Quayle, 1970). Unlike the RMP pathway, the intermediates in the serine pathway are carboxylic acids and amino acids rather than carbohydrates.

The first reaction in the serine pathway (Figure 1.5) involves a chemical condensation between formaldehyde and tetrahydrofolate (THF) to produce N^5, N^{10}-tetrahydrofolate. Serine transhydroxymethylase, catalyses the condensation of the THF derivative with glycine to form serine. The net product of the pathway, phosphoglycerate, is synthesised from two molecules of formaldehyde and one molecule of CO_2. To achieve this, two molecules each of formaldehyde and glycine are converted to two molecules of phosphoglycerate, one of which is converted to phosphoenolpyruvate (PEP). The second important carbon fixation step in the serine pathway is the carboxylation of PEP to oxaloacetate by PEP carboxylase. The CO_2 for this reaction is derived mainly from the oxidation of C_1 substrates. It has been calculated

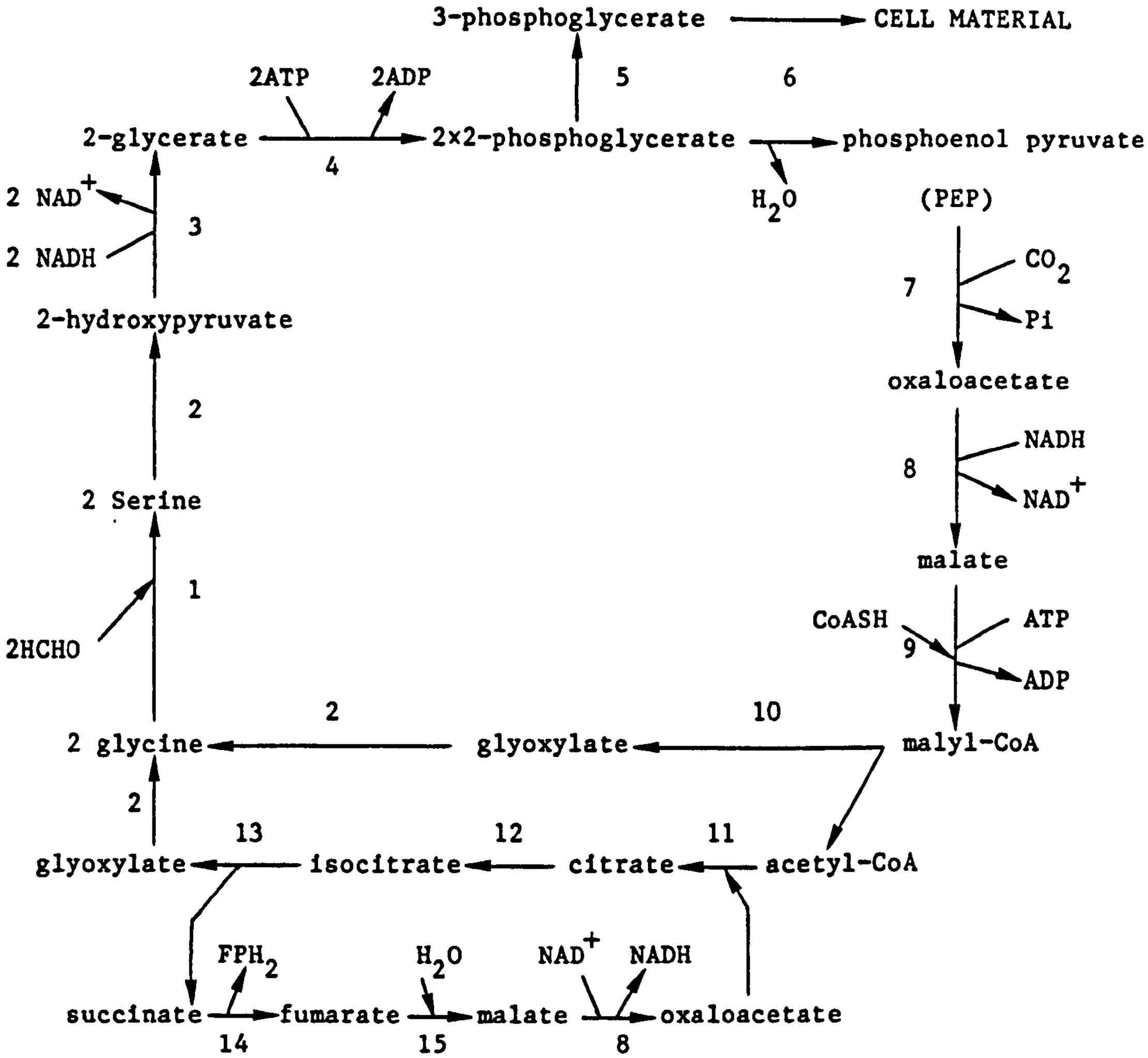


Figure 1.5 THE SERINE PATHWAY (after Anthony, 1982)

1. serine transhydroxymethylase; 2. serine-glyoxylate aminotransferase;

3. hydroxypyruvate reductase; 4. glycerate kinase; 5. phosphoglycerate mutase;

6. enolase; 7. PEP carboxylase; 8. malate dehydrogenase; 9. malate thiokinase;

10. malyl-CoA lyase; 11. citrate synthase; 12. aconitase; 13. isocitrate lyase;

11. succinate dehydrogenase; 12. fumarase.

that 50 % of the carbon derived from methanol is assimilated as CO_2 by the facultative methylotroph, Pseudomonas AM1, using this pathway (Large et al., 1961).

The generation of malyl-CoA from malate and its subsequent cleavage to produce acetyl-CoA and glyoxylate provides another important reaction sequence in the serine pathway. Although M. trichosporium OB3b exhibited Mg^{2+} dependent cleavage of malyl-CoA, no detectable ATP and Coa dependent malate lyase activity was demonstrated (Salem et al., 1973). Indeed, no enzyme has yet been described able to effect the conversion of malate to malyl-CoA in Type II methanotrophs which lack isocitrate lyase.

Serine pathway organisms which lack the enzyme isocitrate lyase face the additional problem of regenerating a second molecule of glyoxylate from acetyl-CoA. The so-called isocitrate lyase negative (ICL^-) pathway has attracted the attention of several research groups in the last twenty years. Although a number of biochemical pathways have been advanced (Anthony, 1975; Kortstee, 1980) to resolve this problem, none have yet been shown to apply to Type II methanotrophs. The most recent addition to this area was that provided by Shimizu and co-workers (1984). They suggested that acetyl-CoA initially condenses with oxaloacetate to form citrate, which is further metabolised via mesaconyl-CoA as described in Figure 1.6. The Shimizu pathway certainly warrants further investigation in Type II methanotrophs and may provide a basis to resolve this long standing metabolic problem.

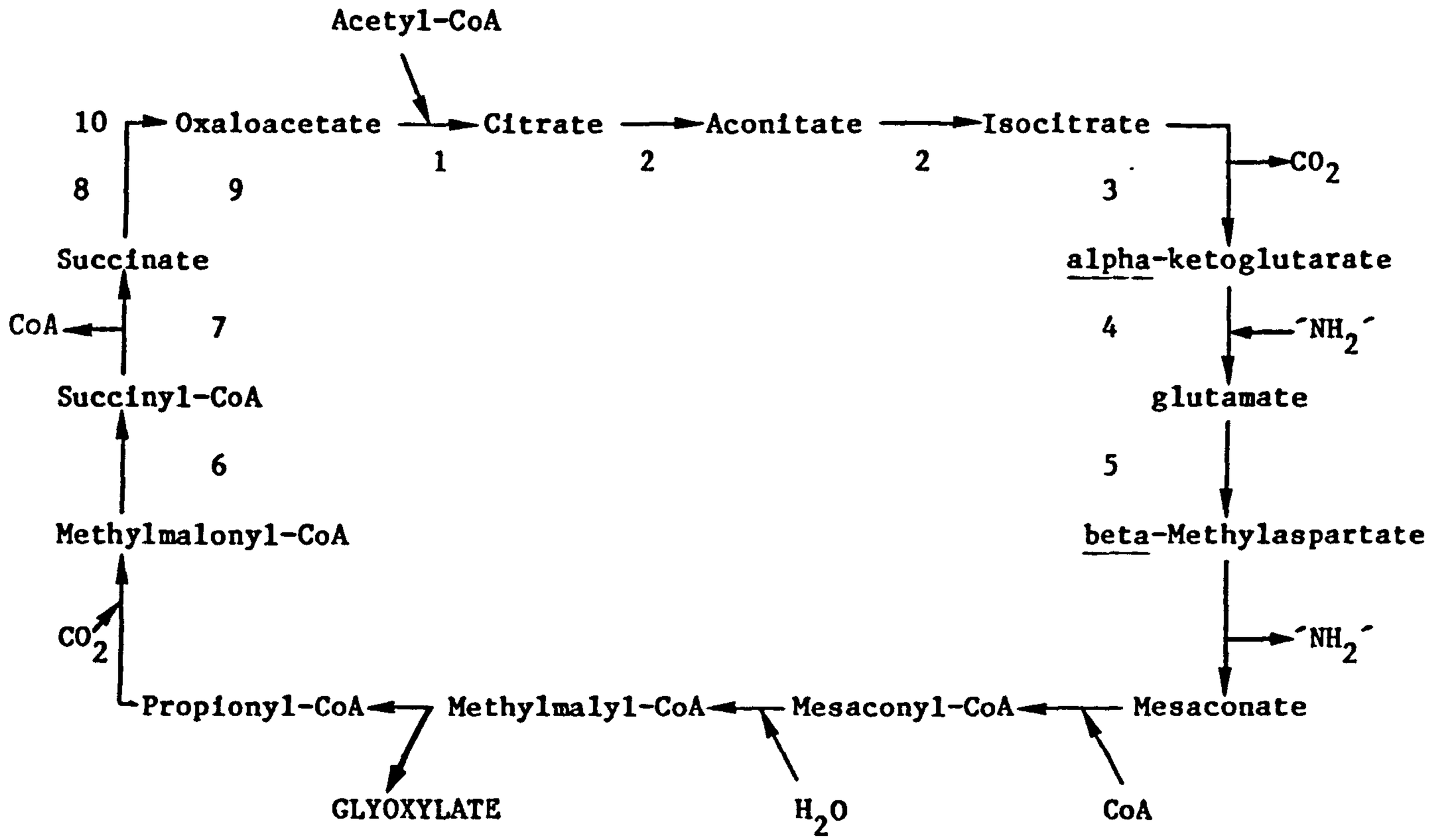
Figure 1.6 The Shimizu Pathway for the Completion of the Serine Pathway in ICL^- Organisms. (Derived from Studies on Protaminobacter ruber; Shimizu et al., 1984).

1. Citrate synthase; 2. Aconitase; 3. Isocitrate dehydrogenase;

4. Transaminase; 5. Glutamate mutase; 6. Methylmalonyl-CoA mutase;

5. Succinyl-CoA synthetase; 6. Succinate dehydrogenase; 9. Fumarase;

10. Malate dehydrogenase.


Acetyl-CoA
10
Oxaloacetate
1
Citrate
2
Aconitate
2
Isocitrate
3
CO_2
8
9
Succinate
alpha-ketoglutarate
CoA
7
4
NH_2
Succinyl-CoA
glutamate
6
5
Methylmalonyl-CoA
beta-Methylaspartate
CO_2
NH_2
Propionyl-CoA
Methylmalyl-CoA
Mesaconyl-CoA
Mesaconate
GLYOXYLATE
H_2O
CoA

1.10 THE TCA IN METHANOTROPHS

One of the most enduring biochemical differences between Type I and Type II methanotrophs is the nature of their tricarboxylic acid (TCA) cycles. In the former, the cycle is incomplete, the crucial enzymic lesion being that of 2-oxoglutarate dehydrogenase, whereas in the latter group the cycle is complete (Davey et al., 1972; Trotsenko, 1976).

The contribution made by the TCA cycle to methanotrophic metabolism is not fully understood. On the one hand it is suggested that the TCA cycle is not particularly important in methanotrophic metabolism (Quayle, 1980) whereas, elsewhere, it is suggested that Type II methanotrophs could not exist without a fully functional TCA cycle due to their high demand for reducing power for MMO and the serine pathway (Whittenbury et al., 1976). The latter argument is, however, unlikely since the activities of the TCA cycle enzymes are probably too low to furnish these requirements (Anthony, 1982).

The role of the TCA cycle has been elucidated by measurement of labelling patterns after the incorporation of radiolabelled acetate into cellular material. In a Type II representative species, Methanomonas methanooxidans, both carbon atoms of acetate were incorporated into amino acids of glutamate and aspartate families as well as those from pyruvate; namely, serine, glycine, alanine, valine, leucine, phenylalanine and tryptophan (Wadzinski & Ribbons, 1975). The liberation of labelled CO_2 during the course of the incubation was consistent with the operation of a complete TCA cycle, performing the

dual roles of biosynthesis and catabolism. In contrast, in a Type I representative species, M. capsulatus (Texas), labelled acetate was incorporated mainly into lipids but 25 % was also shown to reside in only four amino acids, glutamate, proline, arginine and leucine (Patel et al., 1969; Patel et al., 1975). This is indicative of a purely biosynthetic cycle and closely parallels in vitro studies which indicate the enzymic lesion of 2-oxoglutarate dehydrogenase.

The role in which the TCA cycle is employed is thought to be reflected in the mode of regulation of citrate synthase (Weitzman, 1980). Regulation is mediated by feedback inhibition through the "endproduct(s)" of the pathway. When the cycle is operating in its biosynthetic mode control at this site is exerted by one of the two major biosynthetic end products generated by the cycle, namely, 2-oxoglutarate and succinyl-CoA. When the pathway is oxidative, these compounds are intermediates rather than end products and NADH and ATP inhibit the enzyme. Although this corollary appears to hold true in many bacterial groups, the citrate synthases in methanotrophs examined to date (Colby & Zatman, 1975) are insensitive to physiological concentrations of NADH, 2-oxoglutarate and the adenine nucleotides. Certainly, further studies are required in order to rationalise this type of data.

1.11 NITROGEN METABOLISM IN METHANOTROPHS

The division of methanotrophs into two types was originally based on membrane morphology (Whittenbury et al., 1970b) and their carbon assimilation pathways (Lawrence & Quayle, 1970; Davey et al., 1971).

However, a number of recent studies indicate that differences in nitrogen metabolism in methanotrophs may be correlated with the Type I-Type II classification scheme.

The ability to fix nitrogen is characteristic of all Type II methanotrophs studied and is present in only a few Type I organisms, all of which are Methylococcus species, and which may actually belong to a third group, Type X (Baily et al., 1978; Whittenbury & Dalton, 1981; Murrell & Dalton, 1983b). Both glutamate dehydrogenase and glutamine synthetase activities were detected in M. capsulatus (Bath) (Dalton & Whittenbury, 1976), whereas Drozd and co-workers (1977) found that Methylococcus NCIB 11083 only synthesised the enzymes of the glutamine synthetase : glutamine 2-oxoglutarate aminotransferase (GOGAT) system (Brown et al., 1974). A scheme representing the ammonia assimilation pathways in methanotrophs is presented in Figure 1.7.

Shishkina and Trotsenko (1979) compared the pathways of nitrogen metabolism of seven obligate methanotrophs representing both Type I and Type II species. All Type I species, with the exception of M. capsulatus (Foster and Davis strain), have glutamate (or alanine) dehydrogenase and glutamine synthetase activities. The Foster and Davis strain, however, synthesised alanine (but not glutamate) dehydrogenase, glutamine synthetase and, additionally, glutamate synthase; the relative levels of these activities varied depending on the nitrogen source for growth (NH_4^+, NO_3^- or N_2). In contrast, in the Type II species tested, only the enzymes of the GOGAT system could be detected (Shishkina & Trotsenko, 1979; Murrell & Dalton, 1983b; Toukdarian & Lidstrom, 1984).

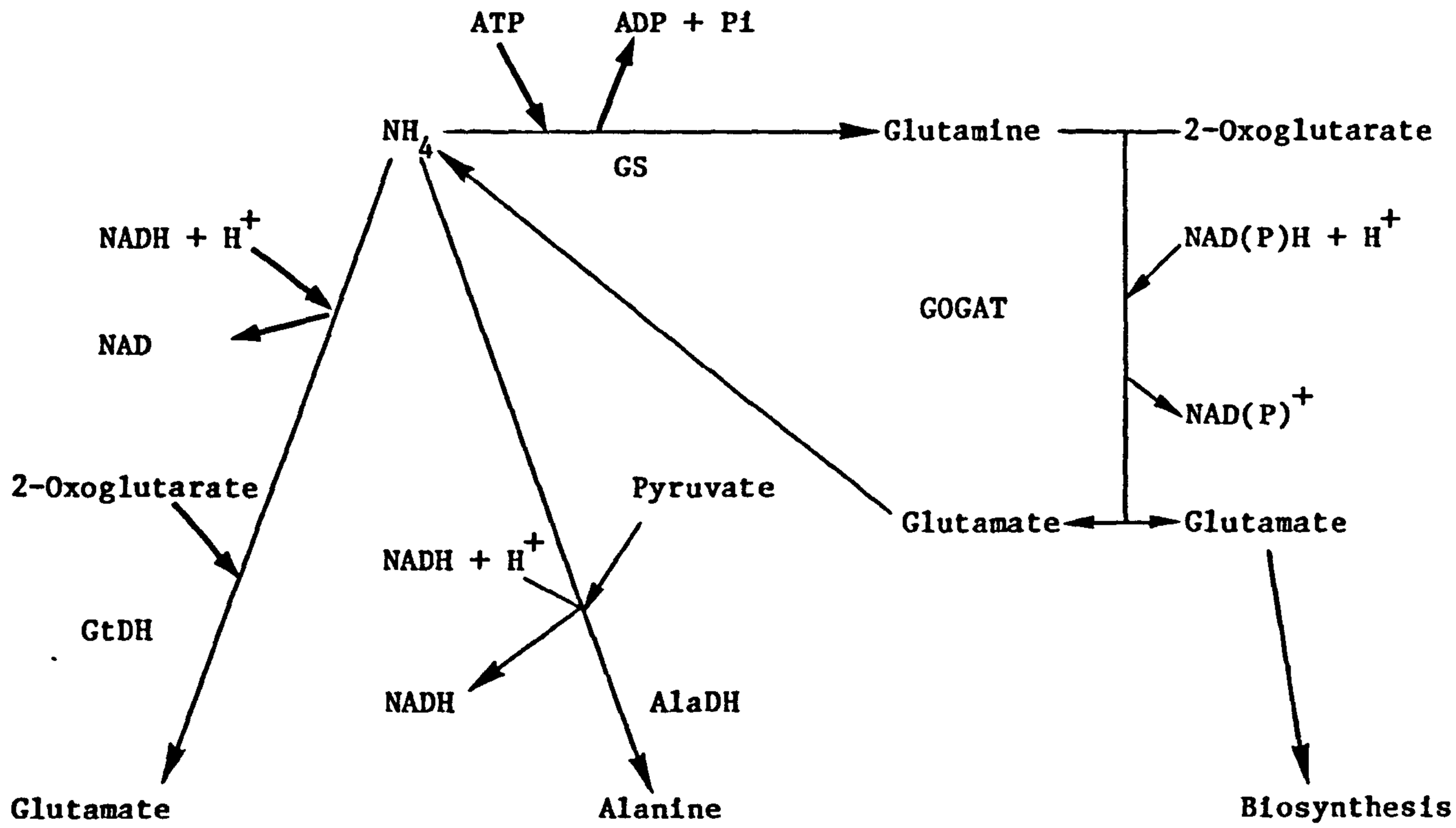


Figure 1.7 Pathways of Ammonia Assimilation (after Dalton, 1979)

Abbreviations: GS, glutamine synthetase; GDH, glutamate dehydrogenase; GOGAT, glutamine(amide):2-oxoglutarate aminotransferase oxidoreductase (glutamate synthase); AlaDH, alanine dehydrogenase.

The regulation of ammonia assimilation in methanotrophs has not been fully examined to date. However, Methylosinus 6 (Toukdarian & Lidstrom, 1984) and M. capsulatus (Bath) (Murrell & Dalton, 1983a) exhibited Mg^{2+}-dependent glutamine synthetase activity which varied with the nitrogen source used for growth. This was probably due to differences in the adenylation state of the enzyme. Adenylation of glutamine synthetase has been demonstrated in many organisms (Gancedo & Holzer, 1968; Tronick et al., 1973; Streicher & Tyler, 1981) and is an important regulatory mechanism in the overall control of the pathway. Nevertheless, studies on other Type II methanotrophs has not given any indication that the enzyme might be adenylated in these organisms (Shishkina & Trotsenko, 1979; Murrell & Dalton, 1983b).

1.12 AIMS OF THIS WORK

The objectives of the work described in this thesis are to investigate the pathway of PHB metabolism in M. trichosporium OB3b and to describe the factors reponsible for its regulation. The work is broadly presented in three parts:

- Characterisation of the enzymes associated with both the synthesis and degradation of PHB, together with a detailed appraisal of their role and regulation in the pathway of polymer metabolism.

- Investigation of the physiological factors responsible for the synthesis of PHB by continuous culture studies.

- Study of *in vivo* metabolite levels during PHB synthesis/mobilsation and relating this data to the information obtained on *in vitro* enzyme regulation in order to postulate an overall control mechanism for polymer metabolism.

CHAPTER TWO

METHODS AND MATERIALS

2.1 MAINTENANCE OF THE ORGANISM

M. trichosporium OB3b, originally obtained from Professor R. Whittenbury (Dept. of Biological Sciences, University of Warwich, Coventry), was maintained on nitrate mineral salts medium (complete autoclavable medium) (Table 2.1) containing agar (1.5% w/v oxoid No 3) in dessicators under a methane/air (1:1, v/v) atmosphere at 30°C. Plates were subcultured every 3-4 weeks.

2.2 GROWTH OF THE ORGANISM

The media used for growth of liquid cultures is detailed in Table 2.1. Sterilisation of media was achieved by autoclaving at 121°C for 15 min. (small scale) or 1 hour (fermenter scale). Methanol was sterilised by filtration using solvent resistant membrane filters (pore size 0.2 µm, Millipore, UK Ltd., Harrow, Middlesex). Sodium bicarbonate solution was sterilised by filtration through an Acrodisc (Gelman Sciences Ltd., Brackmills, Northants).

2.2.1 GROWTH ON METHANOL

Adaption of M. trichosporium OB3b to grow on methanol was achieved by a modification of the method first described by Hou et al. (1979). Growth was initiated from methane grown plate culture. A loopful of organism was transferred to a centre well conical flask (250 ml) containing NMS medium (50 ml) and filter sterilised sodium bicarbonate (0.25% v/v). Methanol (0.5 ml) was supplied in the vapour

		Concentration (g/l)
Salts		
	$NaNO_3$	0.85
	KH_2PO_4	0.56
	Na_2PO_4(.2 H_2O) (.12 H_2O)	0.86 (1.08) (2.17)
	$MgSO_4$.7 H_2O	0.037
	K_2SO_4	0.17
	$CaCl_2$.2 H_2O	0.007
	Fe III EDTA	0.004
Trace Elements		
	$ZnSO_4$.7 H_2O	0.232
	$MnSO_4$.4 H_2O	0.178
	H_3BO_3	0.056
	$CuSO_4$.5 H_2O	0.100
	Na_2MoO_4.2 H_2O	0.039
	$CoCl_2$.6 H_2O	0.042
	KI	0.066
	$FeSO_4$.7 H_2O	0.040
	$NiCl_2$.6 H_2O	0.0004
	0.1 M H_2SO_4	8 ul

2.5 ml of trace elements added per litre of salts

Table 2.1 Nitrate Mineral Salts Medium (NMS) (Taylor, 1983)

phase from the centre well. The flasks were incubated in an orbital shaker (100 rpm) at 30°C until growth occured. Subsequently, aliquots (10 ml) of these cultures were transferred to fresh NMS medium (100 ml) containing methanol (0.5% v/v). Cultures were routinely maintained by transfer to fresh NMS media every three days.

2.3 CONTINUOUS CULTIVATION OF M. TRICHOSPORIUM OB3B

2.3.1 EQUIPMENT

M. trichosporium OB3b was routinely grown in continuous culture in a 5 litre LKB ultraferm fermenter (LKB instruments Ltd., Bromma, Sweden). The unit comprised a stainless steel jacketed base plate incorporating a mechanically sealed stirrer shaft, a pyrex glass cylinder and a stainless steel top plate. Agitation was provided by an impellor attached to the the stirrer shaft and driven by a 1 KWatt motor (0-1500 rpm). The chemostat was used with the following instrumentation and control equipment:

1. Culture pH was monitored by an autoclavable glass combined reference electrode (Russell Ltd., Auchtermuchty, Fife, Scotland) and automatically cotrolled at pH 7.0 by addition of 2 N NaOH or 2 N HCl.

2. Temperature was controlled at 30°C using a thermistor linked to a heating/cooling circuit, consisting of an electrically heated band around the base of the fermenter and a cooling water jacket incorporated into the base plate.

3. Dissolved oxygen tension was monitored by an autoclavable, glass galvanic oxygen electrode (LKB Instruments Ltd., Bromma, Sweden). Prior to inoculation the culture medium was saturated with air and the maximum current output from the electrode recorded as 100% of air saturation; oxygen free nitrogen was passed through the medium to set the oxygen concentration at zero. The oxygen concentration of the culture could be controlled to any value between 0 and 100% air saturation by means of a cotroller.

4. Culture sampling from the fermenter was achieved by means of a sampling port designed to accept 1 oz universals and 500 ml bottles. Contamination was checked by streaking samples onto nutrient agar and incubating at 30^{o}C to allow the growth of any heterotrophs present. Growth was monitored at 600 nm using a DU-8 spectrophotometer (Beckman, RIIC Ltd., High Wycombe, Bucks)

Media, inocula and control lines were of silicone tubing (Gallenkamp Ltd., London) and were introduced into the chemostat by means of stainless steel tubing set into rubber bungs in screw-capped ports in the top plate. Inlet gases were passed through Acro 50 bacteriological filters (Gelman Sciences Ltd., Brackmills, Northants)) prior to admittance into the chemostat. Effluent gases were passed through a condenser (L.H. Engineering Ltd., Stoke Poges, Bucks) to reduce evaporation of the culture.

Inlet gas flow rates were monitored using 'Rotameters' (G.E.C. Elliot Automation Ltd., Croyden, Surrey) and controlled by 'Flowstats'

(G. A. Platon Ltd., Basingstoke, Hants). During continuous culture, medium was introduced into the chemostat by a variable peristaltic pump (LKB Instruments Ltd.). The flow rate of the medium was determined by timing its flow through a vertically clamped pipette (25 ml). Culture effluent flowed from the chemostat via a stainless steel pipe set into the top plate, down into the head space. During this operation the gas outlet, through the condensor, was clamped and excess culture blown out with the gas into a collector pot (20 1).

2.4 HARVESTING

Overflow pots (20 1), derived from chemostat cultures, were harvested by tangential flow ultrafiltration across a Durapore filter membrane (HVLP, 0.5 μm) in a Pellicon cassette system (Millipore, UK Ltd., Harrow, Middlesex). A filtration rate of 0.2 - 0.3 $1.min^{-1}$ was maintained throughout the operation. The concentrated cell suspension (200-300 ml) was centrifuged (10,000 g, 20 min) and washed twice in phosphate buffer (20mM, pH 7.0). The cell paste was either frozen in liquid nitrogen and stored at $-80^{o}C$ or resuspended in appropriate buffer prior to cell breakage.

2.5 PREPARATION OF CELL FREE EXTRACTS OF M. TRICHOSPORIUM OB3B

Whole organism suspensions of M. trichosporium OB3b were disrupted by sonication using a Soniprep-150 sonicator (MSE Scientific Instruments, W. Sussex). Sonication was for 4 x 30 sec. cycles, at an amplitude of 12 - 16 microns, followed by a 2 minute cooling period,

using an ice/water jacket as a coolant. Cell debris and unbroken organisms

were removed by centrifugation (10,000 g, 30 min, 4°C). The supernatant, designated the cell free extract, was carefully removed and stored on ice until required.

2.6 PROTEIN DETERMINATION (after Tan et al., 1984)

The following stock solutions were prepared:

A. 2% (w/v) $CuSo_4$. 5 H_2O

B. 4% (w/v) Na tartrate. $2H_2O$

C. 0.1 M NaOH, 3% Na CO

C* (prepared fresh before use) A (1 ml): B (1 ml): C (48 ml)

D. Folin Ciocalteau reagent (diluted 1:1 with water)

Trichloroacetic acid (TCA) (70 %, 0.1 ml) was added to a protein sample (20 - 100 µg), in a total volume of 0.5 ml, mixed, and stored on ice for 15 min. The mixture was centrifuged (2500 g, 15 min, 4°C), the supernatant discarded, and the precipitated protein rinsed with cold TCA (7% v/v) without mixing. The sample was centrifuged (2500g, 15 min) immediately and the supernatant discarded. The protein was assayed by the modified procedure of Lowry et al. (1951). Water (0.1 ml) and solution C* (0.5 ml) was added to the preciptated protein. The solution was incubated at room temperature (10 min) followed by addition of solution D. The mixture was vortexed immediately and further incubated at room temperature for 30 min. The absorbance of

each sample was measured at 660 nm and referred to a calibration curve constructed from known bovine serum albumin standards

2.7 DRY WEIGHT DETERMINATION

A sample (10 ml) of organisms obtained from continuous or batch culture was centrifuged (2500 g, 15 min), the supernatant discarded and the cell pellet washed in deionised water prior to further centrifugation (2500 g, 15 min). The cell pellet obtained was resuspended in deionised water and dried (105°C) to constant weight.

2.8 ANALYTICAL PROCEDURES

2.8.1 POLY-BETA-HYDROXYBUTYRATE DETERMINATION (after Braunegg et al., 1978)

Cell suspensions (10 ml) were centrifuged (10,000 g, 10 min) and resuspended in acidified methanol (3% v/v H_2SO_4, 1 ml). These suspensions were transferred to screwcapped Reacti-vials (Pierce Chemical Co. Ltd., Rockford, illinois, USA) containing benzoic acid, (400 µg ml^{-1}; internal standard) in acidified methanol (1 ml). The vials were sealed tightly with a teflon coated insert and placed in a water bath at 95°C for 3 hours. After cooling, 1 ml each of chloroform and water was added to each vial and the mixture shaken vigorously (10 min). The chloroform layer, containing the methyl esters of D(-)-3-hydroxybutyrate and benzoate, was analysed by gas chromatography. Aliquots (1 µl) were injected onto a glass column

(2.1 m x 4 mm i.d.) containing 10% Carbowax 20M-TPA on Diatomite C-AW (J.J. Chromatography, Kings Lynn, Norfolk) held isothermally at 165°C, with injector and detector ovens at 200°C and 250°C respectively. Nitrogen carrier gas flow rate was 30 ml. min^{-1}. Appropriate standards of D(-)-3-hydroxybutyrate (50-1000 µg. ml^{-1}) were derivitised by the same procedure and used to construct a calibration table for quantification of peak areas using a computing integrator (Varian CDS 401, Walton on Thames, Surrey).

2.8.2 METHANOL DETERMINATION

Samples obtained from the growth medium (1 ml) were centrifuged, (10,000 g, 10 min., 4°C) to remove cellular material and mixed with propanol (2% v/v; internal standard) in a ratio 1:1 v/v. Aliquots (2 µl) were analysed by gas chromatography on a glass column (1 m x 4 mm i.d) containing 10% Carbowax 20M on Diatomite (J.J Chromatography, Kings Lynn, Norfolk). The column was held isothermally at 110°C with injector and detector operated at 150°C and 200°C respectively. The nitrogen carrier gas flow was maintained at 40 ml. min^{-1}. Quantification of peak height data was determined by a computing integrator, calibrated with the appropriate standards.

2.9 MOLECULAR WEIGHT DETERMINATION

Enzyme molecular weight was estimated by comparison of its elution volume, on a column of Sephacryl S-300 (2.5 x 60 cm), with that of known molecular weight standards (Andrews, 1965). The standard

proteins used as molecular weight markers were as follows: cytochrome C (M_r 12,500 daltons), carbonic anhydrase (M_r 29,000 daltons), bovine serum albumin (M_r 68,000 daltons), alcohol dehydrogenase (M_r 150,000 daltons) and beta-amylase (M_r 200,000 daltons). Blue dextran was used to estimate the void volume of the column. The molecular weight of the native enzyme was determined by comparison with a semilogarithmic plot of molecular weight (standard protein) versus elution volume (Ve).

2.10 SDS-PAGE ANALYSIS OF ENZYME PURITY AND SUB-UNIT MOLECULAR WEIGHT

SDS-Polyacrylamide gel electrophoresis was performed according to Laemlli (1970) in gels of 12% (w/v) acrylamide with a 3% (w/v) acrylamide stacking gel. Samples were electrophoresed at a constant 20 mA until they had reached the stacking gel after which, the current was increased to 30 mA until electrophoresis was complete. Stock solutions prepared are detailed in Table 2.2. Reagent composition of each gel is detailed in Table 2.3. Gels were stained with Coomassie brilliant blue R 250 in water:propan-2-ol:glacial acetic acid (65:25:10 v/v), destained in the above solvent mixture and stored in aqueous acetic acid (7% v/v).

Prior to electrophoresis, protein samples were added to an appropriate amount of sample buffer (Table 2.2) to give a final concentration of 1 mg. ml^{-1} and boiled in sealed eppendorfs (90 sec). The standard proteins used as molecular weight markers were as follows:

Acrylamide Solution	29.2 g Acrylamide 0.8 g N,N´-methylene-bis-acrylamide made up to 100 ml with water
Separating Gel Buffer	18.15 g Trizma base (pH 8.8) made up to 100 ml with water
Stacking Gel Buffer	3.0 g Trizma base (pH 6.8) made up to 100 ml with water
Ammonium Persulphate	100 mg ml^{-1} water
Sample Buffer	4.0 ml distilled water 1.0 ml Tris-HCl (0.5 M, pH 6.8) 0.8 ml glycerol 1.6 ml SDS (10% w/v) 0.4 ml beta -mercaptoethanol 0.2 ml bromophenol blue

Table 2.2 Stock Solutions for SDS-PAGE

(1) Stacking Gel (3% v/v)

	Volume (ml)
Distilled Water	6.1
Tris-HCl (0.5 M, pH 6.8)	2.5
Acrylamide Solution	1.3
SDS (10% w/v)	0.1
Ammonium Persulphate	0.05
TEMED	0.01

(2) Separating Gel (12% v/v)

Distilled Water	13.5
Tris-HCl (1.5 M, pH 8.8)	10.0
Acrylamide Solution	16.0
SDS	0.4
Ammonium Persulphate	0.1
TEMED	0.04

Table 2.3 Contents of Acrylamide Stacking and Separating Gels

albumin, bovine (M_r 66,000 daltons); albumin, egg (M_r 45,000 daltons); glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000 daltons); carbonic anhydrase (M_r 29,000 daltons); trypsinogen (M_r 24,000 daltons); trypsin inhibitor, soybean (M_r 20,100 daltons); alpha-lactalbumin (M_r 14,200 daltons).

2.11 THE SYNTHESIS OF TRIAZINE DYE SEPHAROSE CL-6B CONJUGATES

The Procion dye-Sepharose conjugates were prepared as described by Atkinson (1978). Sepharose CL-6b (Pharmacia, Milton Keynes, Bucks) (20 g in 100 ml of water) was added to the Procion dye (Polysciences, Moulton Park, Northants) (400 mg in 10 ml water) and gently agitated (10 min). This was followed by sodium chloride addition (4 M, 10 ml). The mixture was further agitated (30 min), followed by addition of sodium hydroxide to give a final concentration of 0.01 M. The mixture was incubated (30°C, 2 h) on an orbital shaker (100 rpm). The dye conjugate was washed copiously with (1) water; (2) NaCl (1 M); (3) ethanol (25 % v/v) (4) NaCl (1 M) and stored in phosphate buffer (0.1 M, pH 7.0) at 4°C. The degree of dye substitution was estimated spectrophotometrically following hydrolysis of the gel using acetic acid (7.5 M, 20 min, 80°C).

2.12 IN VIVO METABOLITE STUDIES

2.12.1 SAMPLING PROCEDURE

Samples (10 ml) were withdrawn from the fermenter via a fixed volume Cornwall syringe (Beckton Dickinson, Between Towns Road, Cowley, Oxford) attached to a sample port, whose distal end was in the cell suspension, and quenched in a 1 oz universal containing 1 ml of perchloric acid (60 % v/v). Before collection of the sample, 10 ml of culture, a volume several times greater than the dead space in the sampling line, was expelled to waste. The time taken between ejecting the contents of the dead space to waste and quenching the subsequent sample was less than five seconds.

2.12.2 SAMPLE PREPARATION

After quenching, the sample was stored shortly on ice (10 min) before debris were removed by centrifugation (5,000 g, 15 min). The supernatant was carefully decanted into chilled universal bottles and the pH adjusted to 3.0 by dropwise addition of chilled KOH (5 M) during vortex mixing. The precipitated $KClO_4$ was removed by centrifugation (5,000 g, 15 min) and the supernatant frozen at $-20^{o}C$, prior to freeze drying. The freeze dried cell extracts were resuspended in purified NaH_2PO_4 buffer (100 mM, pH 3.0, 1 ml) and filtered through a 0.2 micron Acro LC13 filter (Gelman Sciences Ltd., Brackmills, Northants)

2.12.3 CHROMATOGRAPHIC APPARATUS

A Varian LC 5000 high performance liquid chromatograph (Varian Associates, Walton on Thames, Surrey) was used for the separation of CoA compounds. The chromatographic system consisted of two pumps used to deliver eluants through a Spherisorb 3 μm ODS column (10 cm x 4.6 mm, Phase Separation, Clwyd, N.Wales) maintained at 30°C by a jacket of circulating water. This was fitted with a guard column (5 cm x 4.6 mm) containing the same packing material. Sample injection was by means of a fixed loop (10-100 μl) and eluant monitored by a fixed wavelength (254 nm) UV detector.

2.12.4 ELUANT PREPARATION AND SEPARATION GRADIENT

The sodium phosphate used to prepare the eluants was purified by passing a stock solution of sodium dihydrogen phosphate (0.5 M) through consecutive columns of anion exchange (AG 1-X8, chloride form) and cation exchange (Chelex 100, sodium form) resins (Bio-Rad Laboratories, Watford, Herts). Each column contained 200 g of resin. Activated charcoal (Sigma Ltd., Poole, Dorset) (14-60 mesh) served as a final column (5 x 25 cm) to remove aromatic contaminants which leached from the ion-exchange resins. The stock solution of phosphate was kept at 4°C and constantly recirculated through these three columns by a peristaltic pump. Buffer A, sodium phosphate (0.2 M, pH 5.0), was prepared from the stock purified phosphate. Buffer B was prepared by mixing phosphate (0.25 M, pH 5.0, 800 ml) with

acetonitrile (200 ml). The buffers were filtered through a 0.2 μm nalgene filter unit (BDH Ltd., Poole, Dorset).

The gradient conditions for the separation of each CoA-compound was as follows:

Time (min)	% Composition of Buffers	
	A	B
0	97	3
2.5	97	3
7.5	82	18
10	72	28
17	55	45
18	55	45
25	10	90
28	10	90
28.1	97	3

Data collection was stopped at 32 min and column reeqilibration was complete after 40 min.

2.12.5 PREPARATION AND QUANTIFICATION OF COENZYME STANDARD SOLUTIONS

Stock solutions of each standard (5 mM) were prepared in sodium phosphate buffer (100 mM, pH 3.0). The millimolar extinction coefficient (E_{LC}) of each standard was determined under the solution

conditions prevailing when it passed from the column and passed through the detector. The coefficient is expressed as follows (equation 2.1):

$$E_{LC} = Em \frac{A_{LC}}{A_m} \quad \ldots(2.1)$$

The millimolar extinction coefficient (Em) was determined at the wavelength of maximum absorbance of the compound. A_{LC} and A_m are the absorbances of the standard at 254 nm under HPLC-elution conditions and at the wavelength of maximum absorbance respectively.

To determine the composition of a standard mixture, the absorbance of the solution was first measured at 254 nm. Then the mixture was separated by HPLC as described above. The amount of any component (a) in the mixture was calculated from equation 2.2 as follows:

$$\text{amount of a (nmol)} = \frac{\%\ a}{100} \times \frac{(A_{254})\text{total} \times \text{cm}^{-1} \times \text{injection volume } (\mu l)}{E_{LC}\ a\ (\mu l/\text{nmol.cm})} \quad \ldots(2.2)$$

where % a is the percentage of compound a in the mixture, (A_{254})total is the absorbance at 254 nm of the mixture determined in a 1 cm light path and E_{LC} a is the millimolar extinction coefficient of compound a at 254 nm.

2.13 MATERIALS

All 'Analar' and 'General Laboratory' grade chemicals were purchased from BDH Ltd., Poole, Dorset; Fisons Ltd., Loughborough, Leicestershire; Koch Light Ltd., Haverhill, Sulffolk. Fine biochemicals, molecular weight markers and analytical enzymes were obtained from Sigma Ltd., Poole, Dorset. Agar (No 3) was obtained from Oxoid Ltd., Basingstoke, Hants.

C H A P T E R T H R E E

THE PURIFICATION AND PROPERTIES OF D(-)-3-HYDROXYBUTYRATE DEHYDROGENASE FROM M. TRICHOSPORIUM OB3B

3.1 INTRODUCTION

All bacteria capable of producing PHB possess a soluble NAD-specific D(-)-3-hydroxybutyrate dehydrogenase (3-HBD) [EC 1.1.1.30]. The enzyme catalyses the reversible oxidation of D(-)-3-hydroxybutyrate in the presence of NAD, to produce acetoacetate and reduced cofactor according to the following reaction (equation 3.1):

$$\text{D(-)-3-Hydroxybutyrate} + \text{NAD} \rightleftharpoons \text{Acetoacetate} + \text{NADH} + \text{H}^{+} \quad \ldots(3.1)$$

NADP or NADPH are unable to replace NAD or NADH in the oxidation and reduction reactions respectively. The enzyme is also highly specific for its substrate in all cases examined to date, although hydroxypentanoate and hydroxyhexanoate are oxidised at low rates by the enzyme from R. spheroides (Bergmeyer et al., 1967).

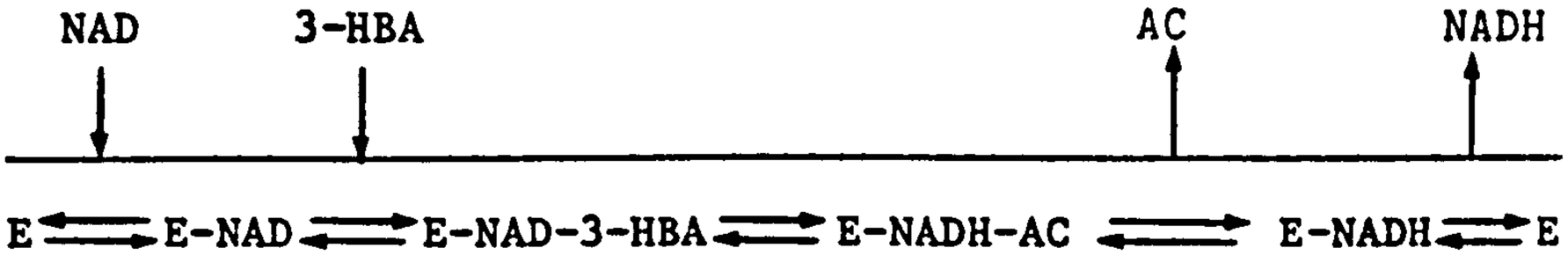
Metal ions have been implicated in both the stability and activation of 3-HBD in many bacteria. A loss in enzyme activity, during purification or extended dialysis without prior addition of a divalent metal ion, has been demonstrated in R. rubrum (Shuster & Doudoroff, 1962), R. spheroides (Bergmeyer et al., 1967), Ps. lemoignei (Delafield et al., 1965a) and Azotobacter vinelandii (Jurtshuk et al., 1968). In the case of R. rubrum and R. spheroides, inhibition of 3-HBD by EDTA could be reversed following incubation with Mg^{2+} and Ca^{2+} respectively. Gavard et al. (1960) have

demonstrated a divalent metal ion dependancy in the activation of 3-HBD from B. megaterium; enzyme activity was optimal in the presence of Mg^{2+} and Mn^{2+}. A similar effect was observed with the enzyme from Z. ramigera I-16-M (Nakada et al., 1981) on addition of Ca^{2+} or Mg^{2+}.

The sensitivity of many 3-HBD's to inactivation by low concentrations of thiol inhibitors (Bergmeyer et al., 1967; Dhariwal & Venkitasubramanian, 1978; Nakada et al., 1981; Kluger & Tsui, 1981) suggests that thiol groups may be involved in the catalytic mechanism of this enzyme. Purified 3-HBD from R. rubrum was shown to contain 5-6 moles of rapidly reacting thiol groups/mole of protein (Bergmeyer et al., 1967) and, furthermore, NADH protects the enzyme from inactivation by mercurials. These results indicate that a thiol group(s) may be present at the active site of the enzyme, because thiols are involved in the binding of nucleotides to proteins (Bergmeyer et al., 1967). In contrast, the cold sensitive 3-HBD from R. rubrum (Shuster & Doudoroff, 1962) does not appear to possess a thiol group essential to the catalytic activity of the enzyme, as judged by its insensitivity to the sulphydryl inhibitors, para-chloromercuribenzoate, sodium arsenite or cadmium chloride.

The 3-HBD-catalysed reaction sequence has been elucidated by analysis of initial velocity double recipricol plots of substrate and product inhibition studies (Hurst et al., 1973; Klugger et al., 1978; Klugger & Tsui, 1981). These studies indicate that the enzyme conforms to the same kinetic pattern of related dehydrogenase enzymes (Tan et al., 1975; Dalziel, 1975) and is consistent with ordered substrate binding (NAD precedes D(-)-3-hydroxybutyrate; acetoacetate leaves

prior to NADH) as indicated in the scheme below (equation 3.2).



$$E \rightleftharpoons E\text{-}NAD \rightleftharpoons E\text{-}NAD\text{-}3\text{-}HBA \rightleftharpoons E\text{-}NADH\text{-}AC \rightleftharpoons E\text{-}NADH \rightleftharpoons E \quad \ldots(3.2)$$

Abbreviations: E, 3-HBD; 3-HBA, D(-)-3-hydroxybutyrate; AC, acetoacetate.

The importance of 3-HBD as a regulatory enzyme in the control of PHB mobilisation was first highlighted by Senior and Dawes (1973). The enzymes isolated from A. beijerinkii, A. eutrophus H16 (Oeding & Schlegel, 1973) and Z. ramigera (Nakada et al., 1981) were each shown to be regulated by the NADH/NAD couple and, in addition, by intermediates of the TCA cycle and/or products of glucose catabolism. In this way, PHB mobilisation would be controlled by feedback inhibition on 3-HBD through metabolic pools linking both oxidative and assimilative metabolism.

3-HBD activity was inducible in the facultative methylotroph, Ps. AM1(Taylor & Anthony, 1976). However, no information regarding its role in the regulation of PHB mobilisation is available in any methylotrophic or methanotrophic species to date. This chapter describes the purification and characterisation of 3-HBD from the obligate methanotroph, M. trichosporium OB3b, and presents a detailed kinetic analysis on its metabolic regulation. Furthermore, the significance of this data in the context of 3-HBD's role in the regulation of PHB mobilisation is assessed and discussed in relation to the intermediary metabolism of this organism.

3.2 EXPERIMENTAL

3.2.1 PROTOCOL FOR THE PURIFICATION OF 3-HBD FROM M. TRICHOSPORIUM OB3B

Frozen cells (120 g) were thawed, resuspended in potassium phosphate buffer (20 mM, pH 7.0 containing dithiothreitol (1 mM), 200 ml) at room temperature. The cell free extract was prepared as described previously (section 2.5), with the exception that the final pH was adjusted to 7.0. Potassium phosphate buffer (20 mM, pH 7.0, containing 1 mM dithiothreitol) was used in each step of the purification sequence. All operations were carried out at $4^{o}C$ where possible.

STEP 1: Chromatography on Procion Red H-3B Sepharose

The crude extract was applied to a column of Red H-3B Sepharose (2.5 x 14 cm). The column was washed with buffer (250 ml) and the enzyme eluted with a linear gradient (300 ml) of potassium chloride (0 - 0.5 M) in phosphate buffer, at a flow rate of 60 $ml.h^{-1}$. Fractions (5 ml) containing enzyme activity were pooled and dialysed by diafiltration (Amicon hollow fibre cartridge, 10,000 molecular weight cut-off), against five volumes of phosphate buffer.

STEP 2: Chromatography on DEAE-Sepharose

The dialysate from the previous step was applied to a column of DEAE-Sepharose (5 x 6 cm). The column was washed with buffer (100 ml)

and the enzyme eluted with a linear gradient (300 ml) of potassium chloride (0-0.4 M) in phosphate buffer, at a flow rate of 60 $ml.h^{-1}$. Active fractions (5 ml) were pooled and dialysed by diafiltration as described above.

STEP 3: Chromatography on Procion Green H-4EBDA

The dialysate was applied to a column of Procion Green H-4EBDA (1.5 x 5 cm) at a flow rate of 60 $ml.h^{-1}$. The column was washed with equilibration buffer (50 ml) and the enzyme eluted in an upwards direction, at a flow rate of 60 $ml.h^{-1}$, with NADH (0.2 mM) in the equilibration buffer (10 ml). Following the application of NADH, the column was washed with equilibration buffer (20 ml) until elution of the enzyme was complete. All enzyme activity was collected, dialysed by diafiltration against phosphate buffer (20 mM, pH 7.0, 10 volumes), and used in subsequent analysis.

3.2.2 ENZYME ASSAYS

The optimum conditions for 3-HBD activity were determined spectrophotometrically at 25°C, in a total reaction volume of 3 ml (1 cm light path).

A. Oxidation reaction (final concentration): Tris-HCl buffer (100 mM, pH 8.2); NAD (0.6 mM); D(-)-3-hydroxybutyrate (10 mM) and purified enzyme (30 ng of protein). The reaction was initiated by the addition of D(-)-3-hydroxybutyrate and followed by measuring the increase in absorbance at 340 nm.

B. Reduction reaction (final concentration): Tris-HCl buffer (100 mM, pH 7.3); NADH (0.1 mM); acetoacetate (3 mM) and purified enzyme (30 ng of protein). The reaction was initiated by the addition of acetoacetate and followed by measuring the decrease in absorbance at 340 nm. One unit of enzyme was defined as the amount of enzyme that, under the conditions of the assay, catalyses the reduction of 1 µmole of NAD in 1 min. at 25°C. During kinetic studies on the reduction reaction (oxidation of NADH), a unit is defined as the amount of enzyme that catalyses the oxidation of 1 µmole of NADH in 1 min. at 25°C).

3.2.3 DETERMINATION OF THE EQUILIBRIUM CONSTANT OF 3-HBD FROM M. TRICHOSPORIUM OB3B

The reaction mixture contained Tris-HCl buffer (100 mM, pH 7.5 - 8.2), D(-)-3-hydroxybutyrate (1 mM), NAD (0.1 mM) and purified enzyme (3 µg) in a total reaction volume of 3 ml. The reaction was initiated by the addition of D(-)-3-hydroxybutyrate and the extinction recorded continuously until equilibrium was reached. Final concentrations of each component at equilibrium were calculated from the change in extinction at 340 nm.

3.3 RESULTS

3.3.1 PURIFICATION OF 3-HBD FROM M. TRICHOSPORIUM OB3B

The protocol devised for the purification of 3-HBD from M. trichosporium OB3b comprises three steps and utilises a combination of triazine dye affinity chromatography and anionic ion-exchange chromatography. The enzyme was prepared in a high yield (72 %) and was shown to be homogeneous by SDS polyacrylamide gel electrophoresis (Figure 3.1). A typical purification scheme is presented in Table 3.1.

A large number of immobilised triazine dyes have been exploited for the purification of proteins by affinity chromatography (Baird et al., 1976; Dean & Watson, 1979; Lascu et al., 1981; Scawen et al., 1983). However, during the development of a protocol for the purification of 3-HBD from M. trichosporium OB3b, only two, Cibacron Blue F3G-A and Procion Red H-3B, were examined for their ability to adsorb the enzyme from the crude extract. Small scale studies, conducted under identical conditions of pH, protein loading and dye substitution on Sepharose CL-6B, showed that both Sepharose-dye conjugates possessed a high affinity for 3-HBD. The binding capacity of Procion Red H-3B (8 +/- 0.8 units of 3-HBD/ml dye-Sepharose) for 3-HBD however, was markedly higher than Blue F3G-A (5 +/- 0.3 units of 3-HBD/ml of dye-Sepharose) as was the level of purification achieved, following its elution using a salt gradient. In view of its high binding capacity for 3-HBD, together with the excellent purification and yield obtained, Procion Red H-3B was subsequently chosen as the

STEP	VOLUME (ml)	PROTEIN (mg/ml)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)*	SPECIFIC ACTIVITY (µmol/min/mg)	PURIFICATION FACTOR (fold)	YIELD (%)
Cell free extract	222	12.1	2686	483	0.18	1	100
Red H-3B Sepharose	145	0.34	49.3	414	8.40	46.7	85
DEAE-Sepharose	51	0.34	17.1	377	21.96	122.7	78
Green H-4EBDA	42	0.026	1.1	350	327.50	1819	72

* 1 unit = 1 µmole NAD reduced min^{-1} at $25^{o}C$

Table 3.1 Purification of 3-HBD from M. trichosporium OB3b

The table shows a summary of a typical purification from 120g of frozen cell paste. Experimental details are described in section 3.2.1

Figure 3.1 Sodium Dodecyl Sulphate Polyacrylamide (SDS) Gel Electrophoresis of 3-HBD from M. trichosporium OB3b

Samples from each step in the purification sequence were electrophoresed on 12 % gels by the method of Laemmli (1970) (section 2.10). Protein staining was performed with Coomassie brilliant blue.

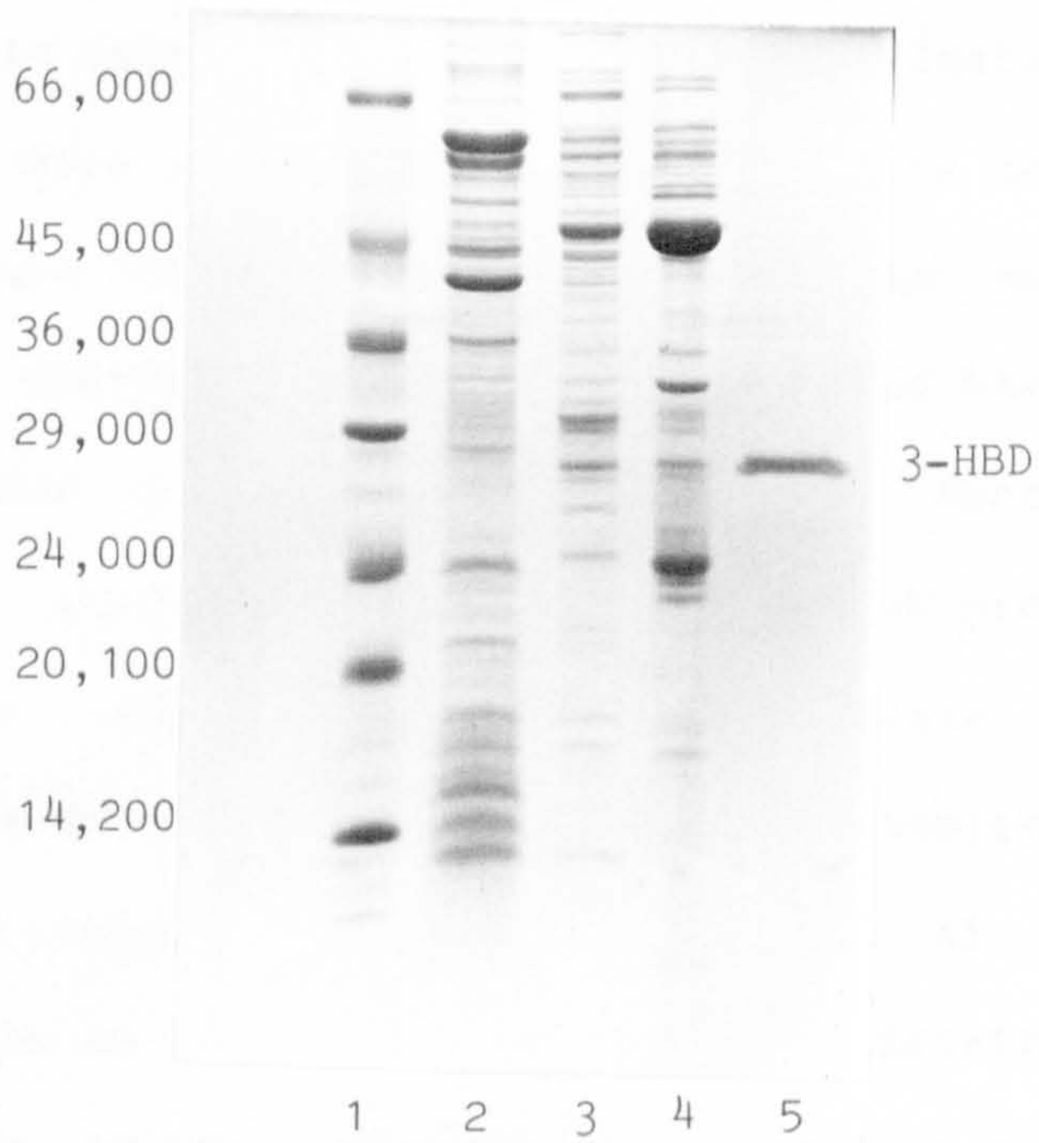
Lane 1, molecular weight markers (25 µg);

Lane 2, crude extract (25 µg);

Lane 3, Procion Red H-3B eluate (25 µg);

Lane 4, DEAE-Sepharose eluate (25 µg);

Lane 5, Green H-4EBDA eluate (10 µg).


66,000
45,000
36,000
29,000
24,000
20,100
14,200
3-HBD
1
2
3
4
5

first chromatographic step in the purification sequence.

As with any conventional affinity chromatography the success of interaction between the immobilised dye and the enzyme will largely depend on the experimental conditions chosen (Lowe, 1979). Therefore, a number of operational parameters such as protein loading, pH and cofactor eluant were investigated in order to optimise 3-HBD resolution from "contaminating proteins" on Red H-3B Sepharose. Of those variables tested, the protein loading was found to be the most significant factor in determining the level of purification achieved; the best results were obtained by utilising the maximum binding capacity of the column for 3-HBD. This observation is not altogether surprising if we consider that each protein would have a different binding affinity for the triazine dye ligand. Hence, following saturation of the binding sites on the column with protein, further application of crude extract would serve to displace low affinity proteins with those which have a higher binding affinity for the dye. This technique introduces further fractionation of 3-HBD from contaminating protein on the basis of its binding affinity for the dye ligand. Subsequently, during a relatively non-specific salt elution of 3-HBD, only proteins with a similar or higher affinity for the dye should theoretically be eluted. Proteins with a lower affinity for the dye than 3-HBD would have been displaced previously.

The pH at which 3-HBD was bound to Red H-3B had a marked effect on both the elution characteristics of the enzyme and the level of purification achieved. At pH 6.0 the enzyme was strongly adsorbed to the column, such that its activity, following elution, peaked at 200

mM KCl, while at pH 7.0 elution was optimal at 125 mM KCl. One possible explanation for this observation is that pH 6.0 is lower than the isoelectric point of 3-HBD and that there is an increased electrostatic interaction between the enzyme and the negatively charged sulphonate groups on the aromatic rings of the dye molecule. Therefore, Red H-3B appears to act as an ion-exchanger in an analogous fashion to that described for Cibacron Blue F3G-A (Lascu et al., 1984). In order to maximise the yield and ease of elution of 3-HBD from Red H-3B a pH of 7.0 was maintained throughout this process.

The ligand concentration has been shown to effect both protein adsorption and desorption processes on the affinity matrix (Watson et al., 1978; Scawen et al., 1982). Generally, protein adsorption is proportional to the degree of dye substitution up to a fixed value (2-4 μmol of dye/ml of Sepharose) above which the capacity of the affinity matrix decreases, probably as a consequence of steric hindrances due to the presence of a large number of dye molecules. In this study a dye substitution value of 4 μmol/ml Sepharose was utilised for 'large scale' purification of the enzyme. Although the optimum dye substitution on Sepharose CL-6B was not fully investigated during this study, this level of substitution combined a high binding capacity for the enzyme with ease of elution and high recovery. The elution profile for 3-HBD from Procion Red H-3B is presented in Figure 3.2. The elution profile from DEAE-Sepharose, which is the second step in the purification sequence, is shown in Figure 3.3.

Since triazine dyes can mimic NAD, NADP or ATP and also behave as strong cationic ion-exchangers, the potential range of proteins which

Figure 3.2 Elution Profile of 3-HBD from Procion Red H-3B Sepharose

The crude extract (2.8 g of protein) was applied to a column of Procion Red H-3B Sepharose (2.5 x 14 cm) equilibrated with phosphate buffer (20 mM, pH 7.0) containing dithiothreitol (1 mM). Elution was performed with a linear gradient of KCl (0 - 0.5 M in equilibration buffer, 300 ml) at a flow rate of 60 ml.h^{-1}. Fractions of 5 ml were collected. (●) 3-HBD activity; (○) protein (mg.ml^{-1}); (--) KCl (M).

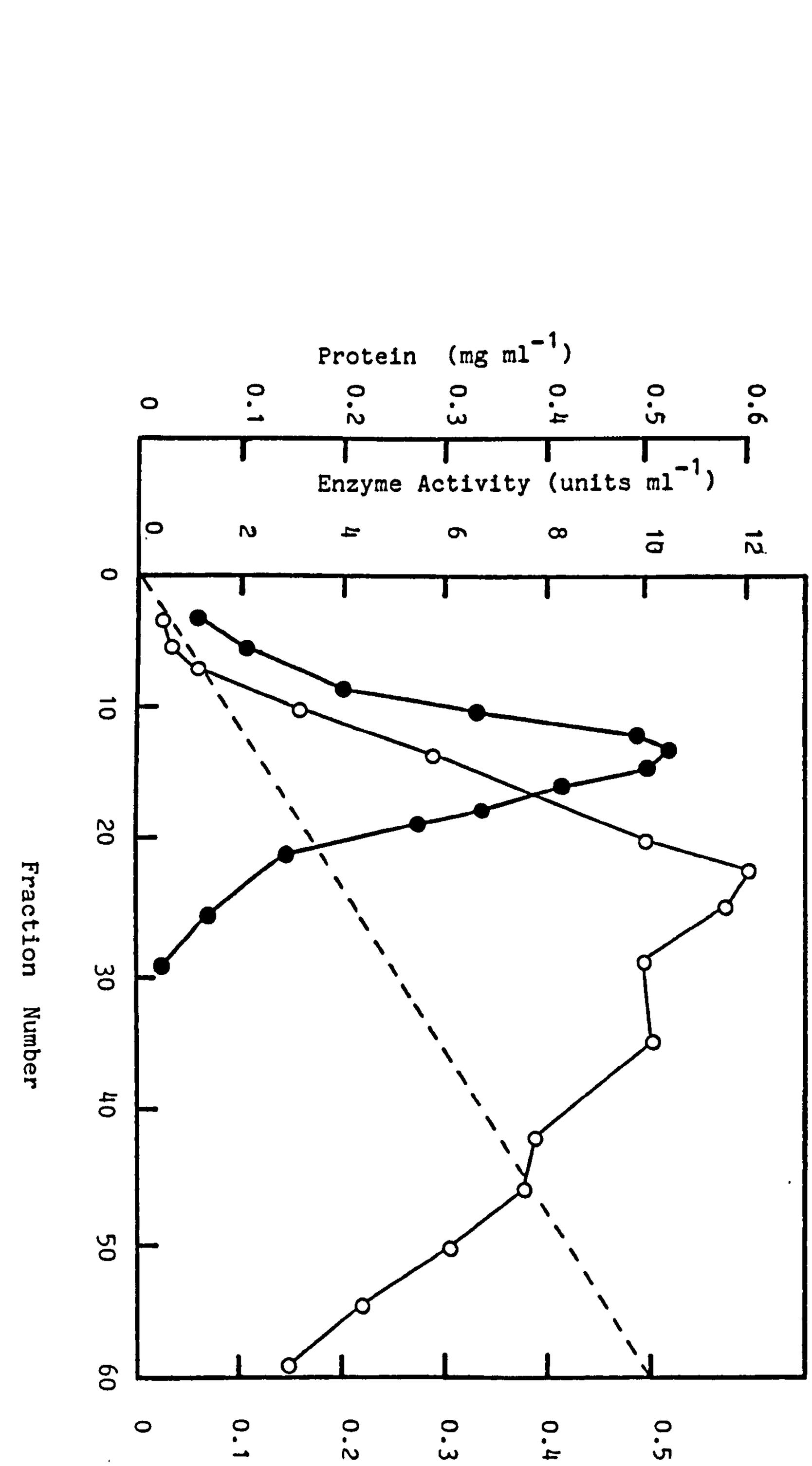
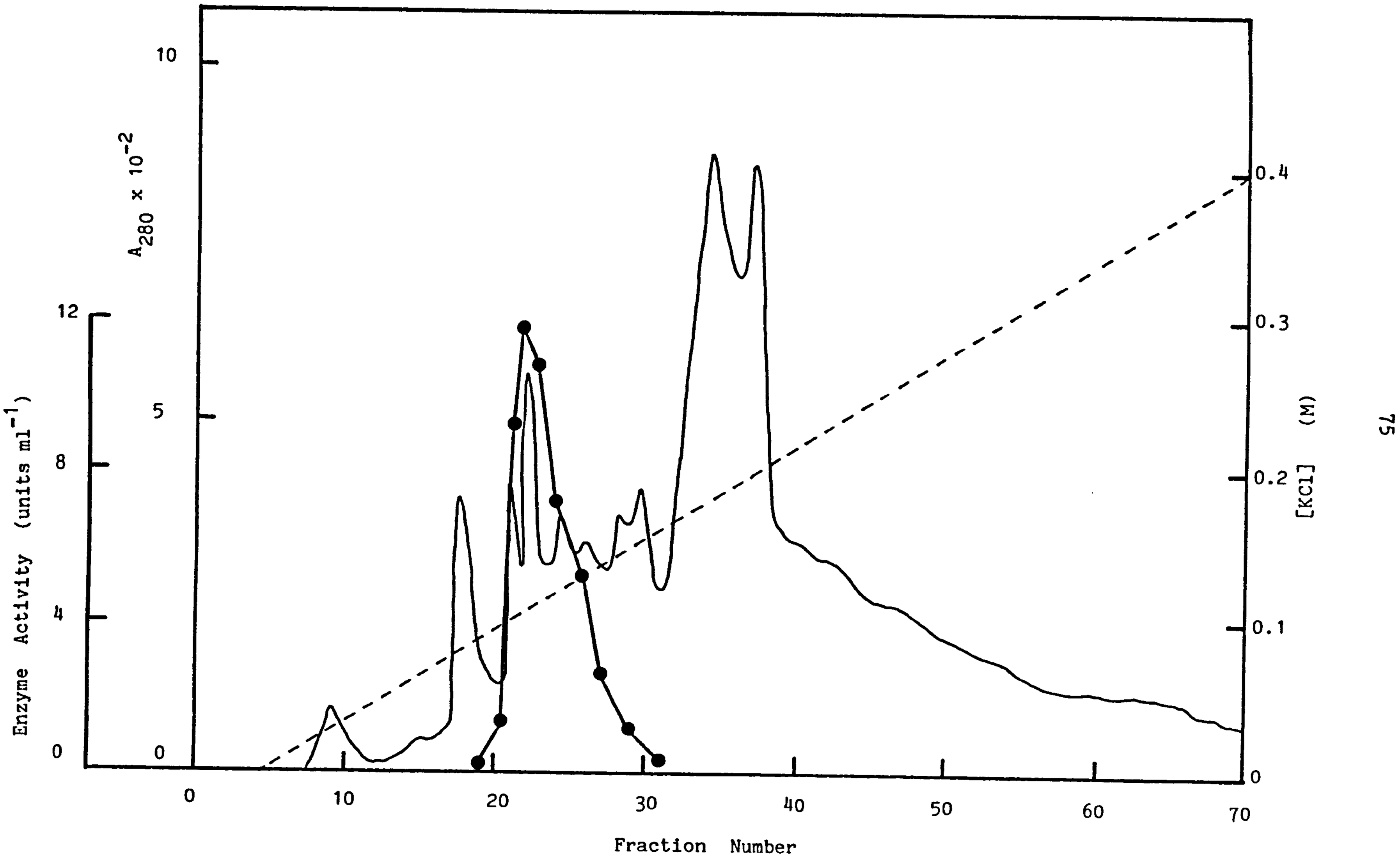

Protein (mg ml^{-1})
0
0.1
0.2
0.3
0.4
0.5
0.6
Enzyme Activity (units ml^{-1})
0
2
4
6
8
10
12
Fraction Number
0
10
20
30
40
50
60
[KCl] (M)
0
0.1
0.2
0.3
0.4
0.5

Figure 3.3 Elution Profile of 3-HBD from DEAE-Sepharose

3-HBD was applied to a column of DEAE-Sepharose (5 x 6 cm) equilibrated with phosphate buffer (20 mM, pH 7.0) containing dithiothreitol (1 mM). Elution was performed with a linear gradient of potassium chloride (0 - 0.4 M, in equilibration buffer, 300 ml) at a flow rate of 60 ml. h^{-1}. Fractions of 5 ml were collected. (●) 3-HBD activity; (--) KCl (mM); (—) A_{280}.


10
12
$A_{280} \times 10^{-2}$
5
8
4
0
0
Enzyme Activity (units ml^{-1})
0.4
0.3
0.2
0.1
0
[KCl] (M)
0
10
20
30
40
50
60
70
Fraction Number

they bind is extremely wide (Haff & Easterday, 1978). Nevertheless, a high degree of selectivity in the purification of the requisite protein can be introduced by specific cofactor elution. Following its initial purification, 3-HBD could be eluted selectively from Cibacron Blue F3G-A, Procion Red H-3B and a third triazine dye, Green H-4EBDA, on addition of either NADH or NAD to the equilibration buffer. In each case, both cofactors were similarly effective in producing a homogeneous enzyme preparation, the only difference being the concentration of cofactor required to initiate desorption of the enzyme from each ligand. In this respect, the concentration of NADH required was approximately 50 % less than NAD in each case. One possible explanation is the affinity of 3-HBD for its cofactors (Section 3.3.4), which is highest in the case of NADH. Increasing the concentration of the requisite cofactor essentially diminishes the binding affinity of the enzyme for the ligand in a competitive fashion until its desorption occurs.

The choice of Green H-4EBDA as the final chromatographic step in the purification sequence was due entirely to the low level of NADH required (0.05 mM NADH) to initiate elution of 3-HBD from the matrix. Cibacron Blue and Red H-3B required higher concentrations of cofactor to effect the same desorption (0.1 mM and 0.15 mM NADH respectively). This is possibly a reflection of the difference in binding affinity of each triazine dye for 3-HBD.

3.3.2 MOLECULAR WEIGHT OF 3-HBD

The molecular weight of the native enzyme was determined by comparison of its elution volume (Ve), after chromatography on Sephacryl S-300, with the values obtained from proteins of known molecular weight (Section 2.7). A semilogarithmic plot of the elution volumes of the standard proteins produced a straight line (Figure 3.4) and by extrapolating the value of the elution volume (217 ml) to the abscissa a native molecular weight of 28,000 was obtained.

The subunit molecular weight of the enzyme was determined by comparing its relative mobility (R_f), on a 12 % SDS polyacrylamide gel with those of standard proteins (Section 2.8). From this data (Figure 3.5) a protein band corresponding to a molecular weight of 26,000 indicated that the enzyme consisted of a single monomeric chain.

3.3.3 STABILITY OF 3-HBD

3-HBD remained stable in the cell free extract with no detectable loss of activity over a period of 72 h at 4°C. Progressive purification, however, yielded an unstable enzyme, such that the post DEAE-Sepharose enzyme preparation lost 50 % activity in 24 h at 4°C, with complete inactivation on freezing. The influence of a number of protective agents on stability were examined and their effects summarised in Table 3.2. The cations tested afforded no protection, whereas NADH, NAD and the sulphydryl reagents, dithiothreitol and 2-mercaptoethanol, completely stabilised enzyme activity. Nevertheless, the enzyme was still largely inactivated on freezing

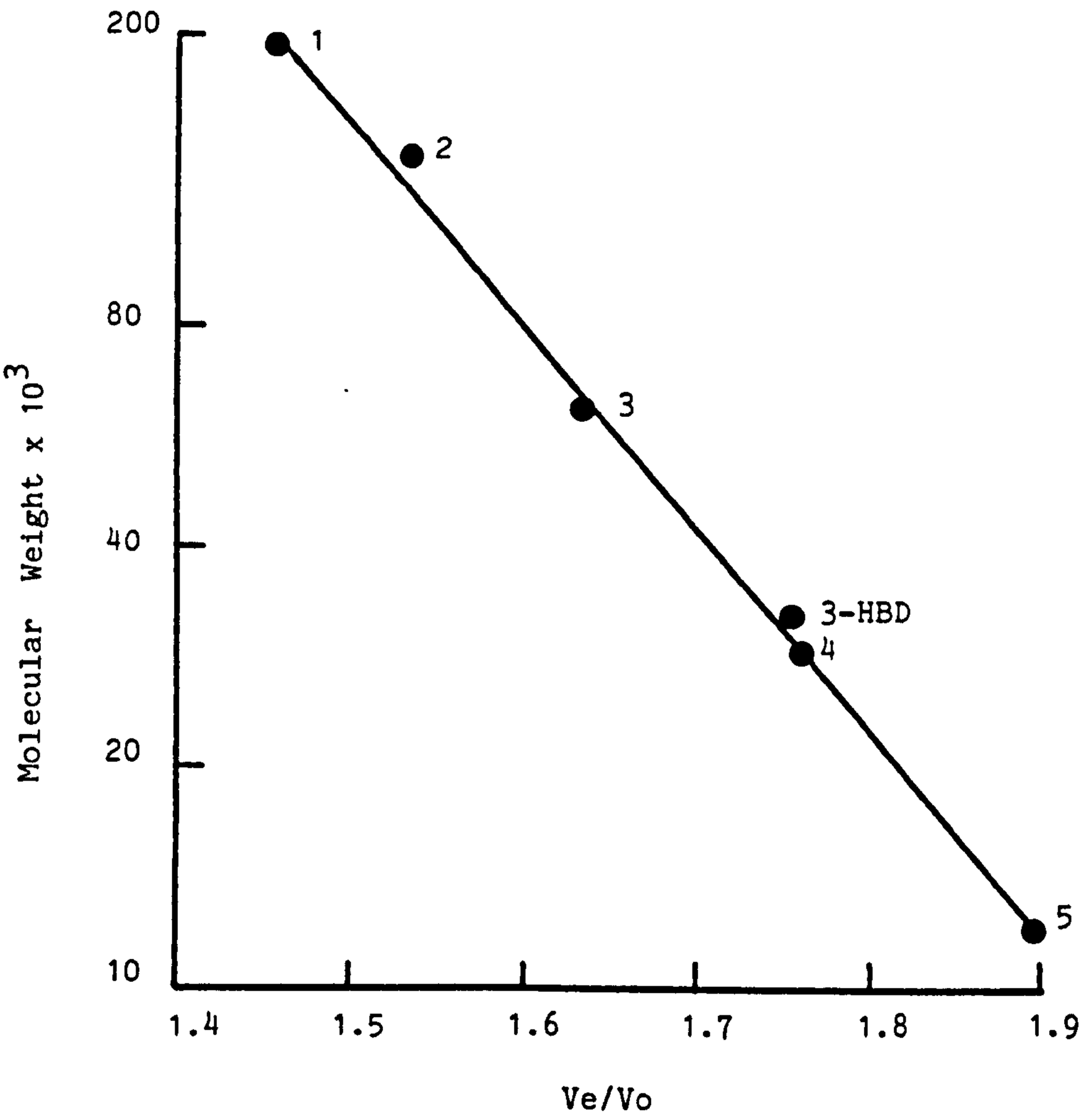


Figure 3.4 Determination of the Molecular Weight of 3-HBD from M. trichosporium OB3b by Sephacryl S-300 Gel Filtration

The elution volume of 3-HBD on a column of Sephacryl S-300 was compared to proteins of known molecular weight. 1. Beta-Amylase; 2. Alcohol Dehydrogenase; 3. Albumin, Bovine; 4. Carbonic Anhydrase; 5. Cytochrome C. (See section 2.9 for further details).

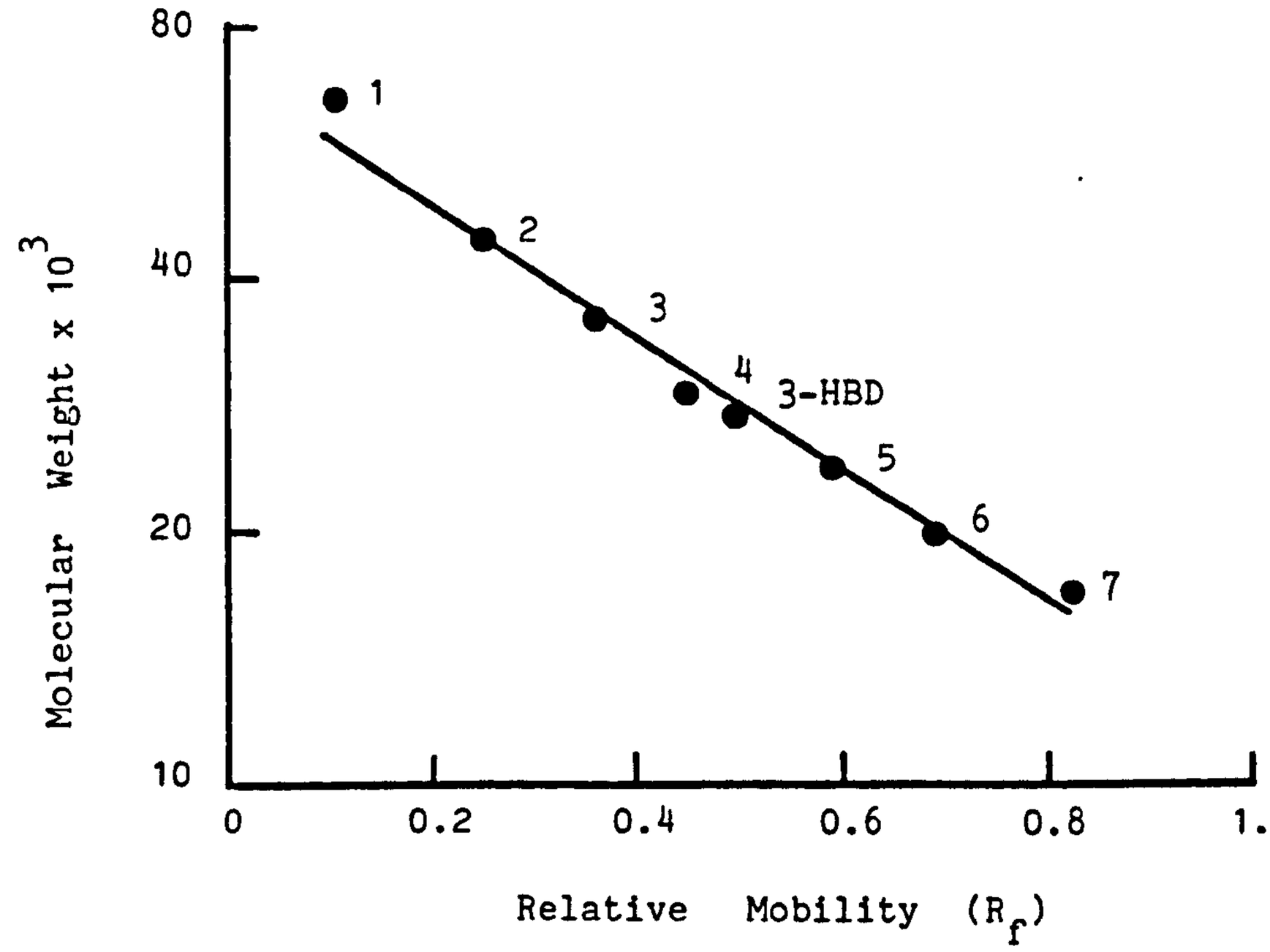


Figure 3.5 Determination of the Subunit Molecular Weight of 3-HBD from M. trichosporium OB3b by SDS Polyacrylamide Gel Electrophoresis

The relative mobility of the dissociated enzyme on a 12 % polyacrylamide gel, containing SDS, was compared to proteins of known molecular weight. 1. Albumin, Bovine; 2. Albumin, Egg; 3. Glyceraldehyde-3-phosphate Dehydrogenase; 4. Carbonic Anhydrase; 5. Trypsinogen; 6. Trypsin Inhibitor, Soybean; 7. Alpha-Lactalbumin. (See section 2.10 for further details).

ADDITION	FINAL CONCENTRATION (μM)	ACTIVITY (%)
Dithiothreitol	1000	95
Beta-Mercaptoethanol	5000	87
NADH	500	95
NAD	500	90
Cu^{2+}	5	35
Ca^{2+}	5	48
Mg^{2+}	5	39
Mn^{2+}	5	49
Ni^{2+}	5	48
Blank	no addition	42

Table 3.2 Effect of Various Cations and Stabilising Agents on the Activity of 3-HBD from M. trichosporium OB3b

The incubation mixture contained protective agent to be tested, enzyme (2.5 μg of protein) obtained from post Red H-3B Sepharose eluate, and phosphate buffer to a final volume of 0.5 ml. Samples were incubated at $4^{o}C$ and activity determined after 24 h. The results are expressed as a percentage of the initial activity.

even with prior addition of each of the reagents shown to stabilise activity in solution. Long term storage of the enzyme was best achieved at -80°C by prior precipitation with ammonium sulphate. Under these conditions, the enzyme appeared to lose less than 25 % activity following six months storage.

3.3.4 CATALYTIC PROPERTIES OF 3-HBD

The pH profiles for the oxidation and reduction reactions are presented in Figures 3.6.1 and 3.6.2 respectively. In both cases the pH optima exhibited a broad range when assessed in Tris-HCl and phosphate buffers. Oxidation was optimal in the range of pH 7.1 - 8.8 and reduction was optimised in the range 6.0 - 7.4. All further kinetic analysis on the oxidation reaction was conducted in Tris-HCl buffer, pH 8.2.

The kinetic constants for the oxidation and reduction reactions were calculated by the method of Lineweaver and Burk (1934) and represent mean values from two homogeneous preparations of the enzyme.

The K_m values for D(-)-3-hydroxybutyrate (Figure 3.7) and NAD (Figure 3.8) were found to be 1.33×10^{-3} M and 0.062×10^{-3} M respectively, whereas, the maximum rate, V_{max}, of D(-)-3-hydroxybutyrate oxidation was 325 units $(mg.\ protein)^{-1}$. NADP could not replace NAD in the reaction sequence when tested over a range of concentrations (0 - 2 mM).

Figure 3.6 Effect of pH on 3-HBD Activity from M. trichosporium OB3b

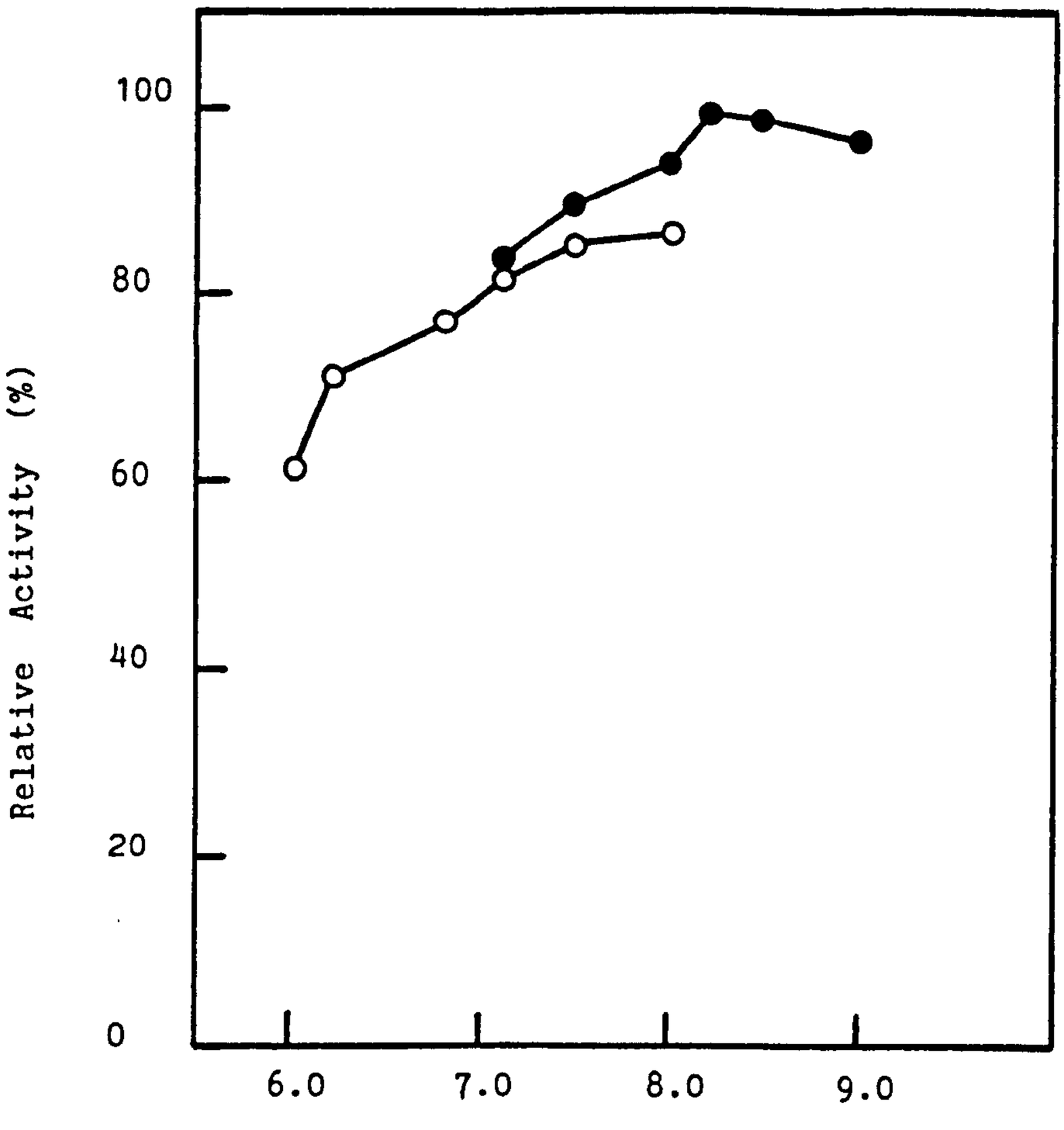
The composition of the reaction mixture was described in section 3.2.2 with addition of purified enzyme solution (30 ng). Buffers (100 mM) used were:

(O) Phosphate; (●) Tris-HCl.

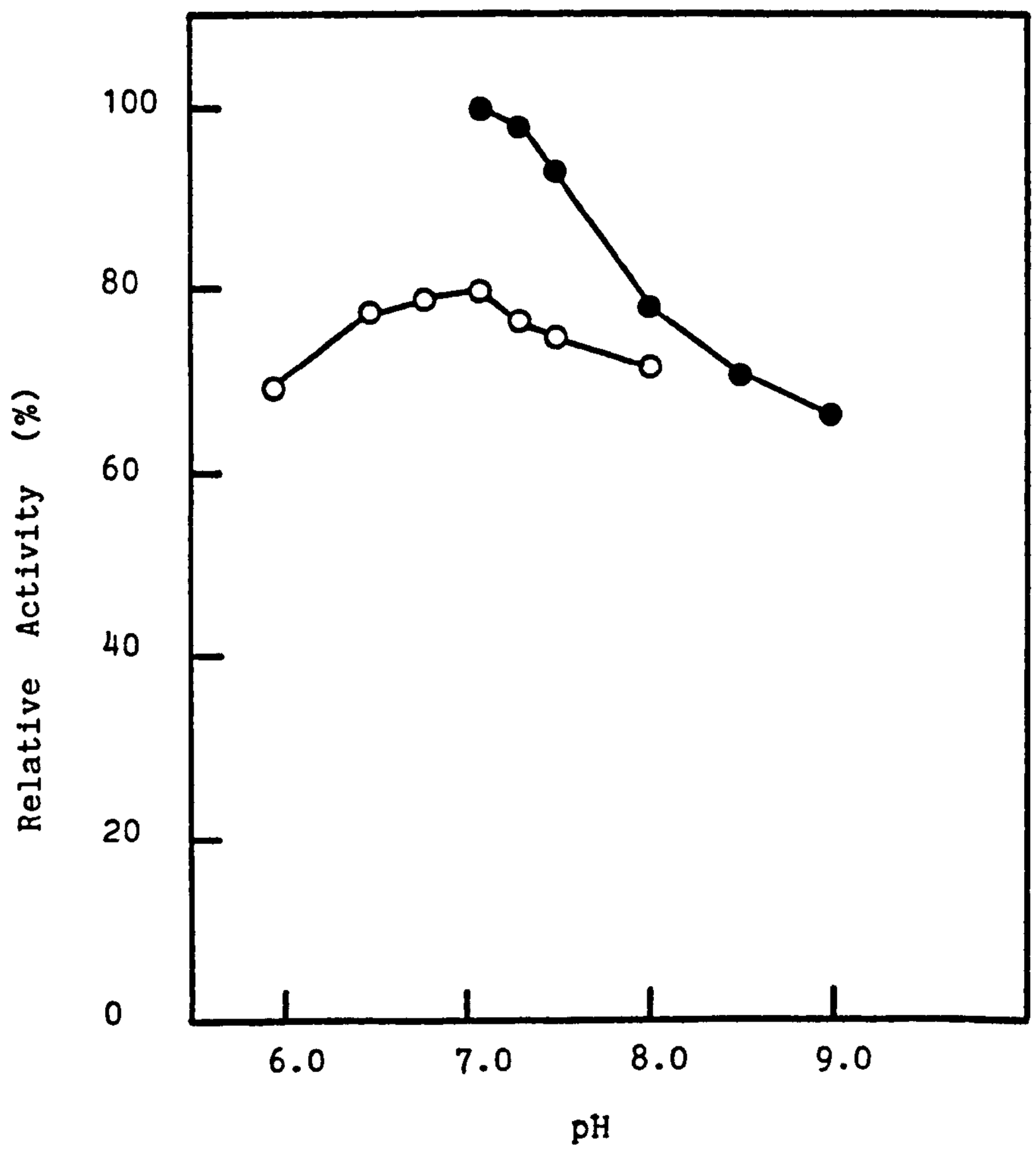
(1) D(-)-3-Hydroxybutyrate oxidation.

(2) Acetoacetate reduction.

(1)


100
80
60
40
20
0
Relative Activity (%)
6.0
7.0
8.0
9.0
pH

(2)


100
80
60
40
20
0
Relative Activity (%)
6.0
7.0
8.0
9.0
pH

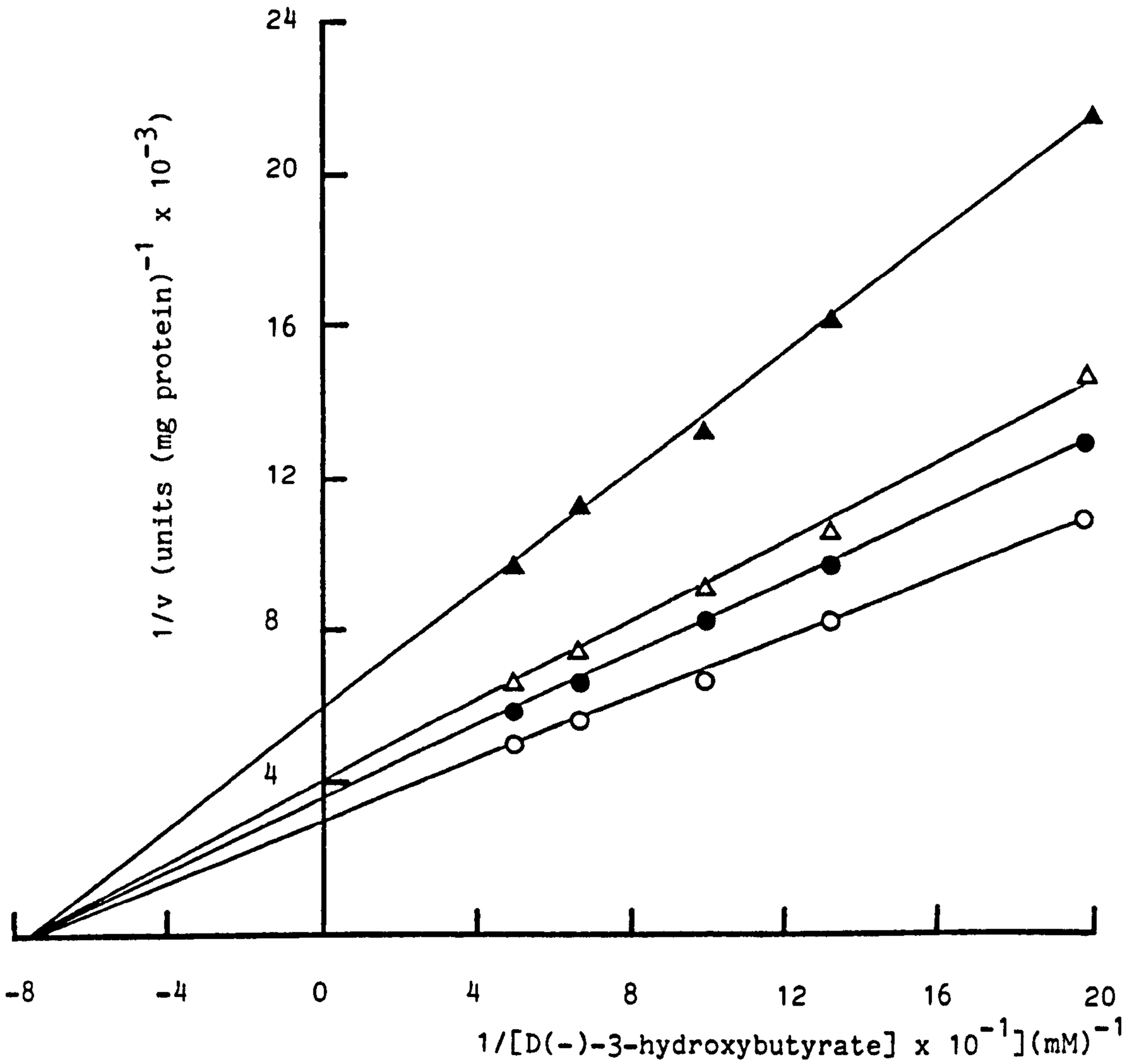


Figure 3.7 Lineweaver-Burk Transformation of Kinetic Data for the Oxidation of D(-)-3-Hydroxybutyrate Catalysed by 3-HBD at Different Initial Levels of NAD.

Cuvettes contained, in a total reaction volume of 3 ml: Tris-HCl buffer (100 mM, pH 8.2); D(-)-3-hydroxybutyrate (0.75 - 2.0 mM); purified enzyme solution (30 ng of protein). Concentration of NAD (mM): (○) 0.6; (●) 0.15; (△) 0.1; (▲) 0.05.

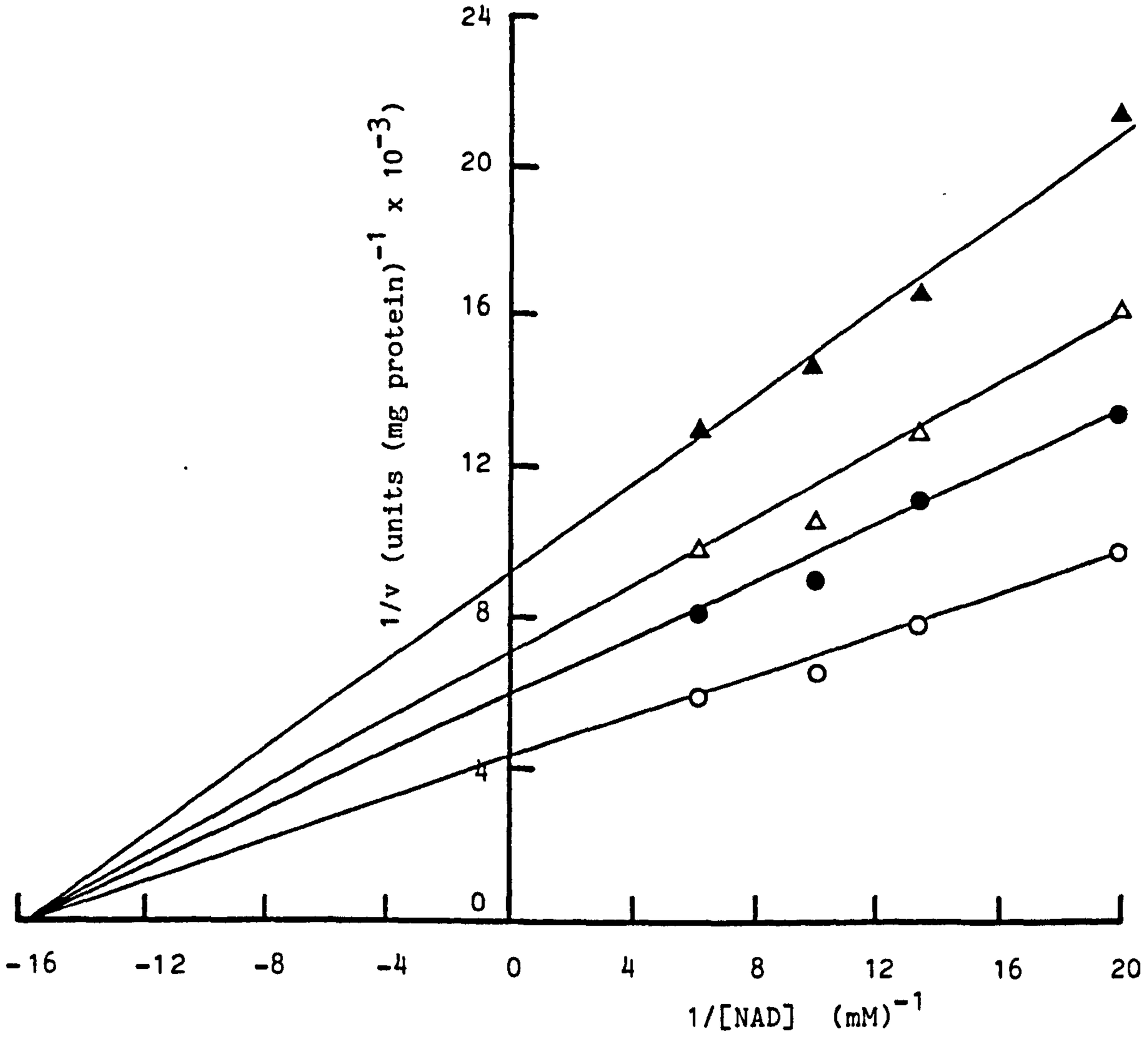


Figure 3.8 Lineweaver-Burk Transformation of Kinetic Data for the Reduction of NAD, Catalysed by 3-HBD at Different Initial Levels of D(-)-3-Hydroxybutyrate

Cuvettes contained in a total reaction volume of 3 ml: Tris-HCl buffer (100 mM, pH 8.2) NAD (0.05 - 0.6 mM); purified enzyme solution (30 ng of protein). Concentration of D(-)-3-hydroxybutyrate (mM):

(○) 2.0; (●) 1.0; (△) 0.75; (▲) 0.5.

The Km values for acetoacetate (Figure 3.9) and NADH (Figure 3.10) were 0.13 x 10^{-3} M and 0.034 x 10^{-3} M respectively in Tris-HCl buffer (pH 7.3). The maximum rate, V_{max}, of acetoacetate reduction by NADH was 196 units (mg. protein)$^{-1}$.

3.3.5 THE EFFECT OF POTENTIAL INHIBITORS ON 3-HBD

In order to assess the possible biochemical regulation of 3-HBD and its relationship to the mobilisation of PHB in M. trichosporium OB3b, the purified enzyme was tested with a range of potential effectors, up to a concentration of 10 mM (unless stated). During this study, non-saturating concentrations of both D(-)-3-hydroxybutyrate (0.5 mM) and NAD (0.025 mM) were employed, such that any competitive inhibition of the enzyme was not masked by saturation with the substrate (or cofactor) of the reaction.

Among the groups tested were the adenosine phosphates (ATP, ADP and AMP), nicotinamide nucleotides (NADH, NADP and NADPH, up to 2 mM), intermediates of the TCA cycle, precursors and products of PHB metabolism (acetyl-CoA, acetoacetyl-CoA, D(-)-3-hydroxybutyryl-CoA, CoASH and acetoacetate) and several intermediates of the serine pathway (serine, glyoxylate, hydroxypyruvate and 3-phosphoglycerate). Of those tested, inhibition was observed in the presence of NADH and acetoacetate only, the products of the oxidation reaction sequence. No inhibition was detected in the presence of the other groups tested.

A preliminary study on the kinetics of the reduction reaction showed that it was susceptible to both substrate inhibition

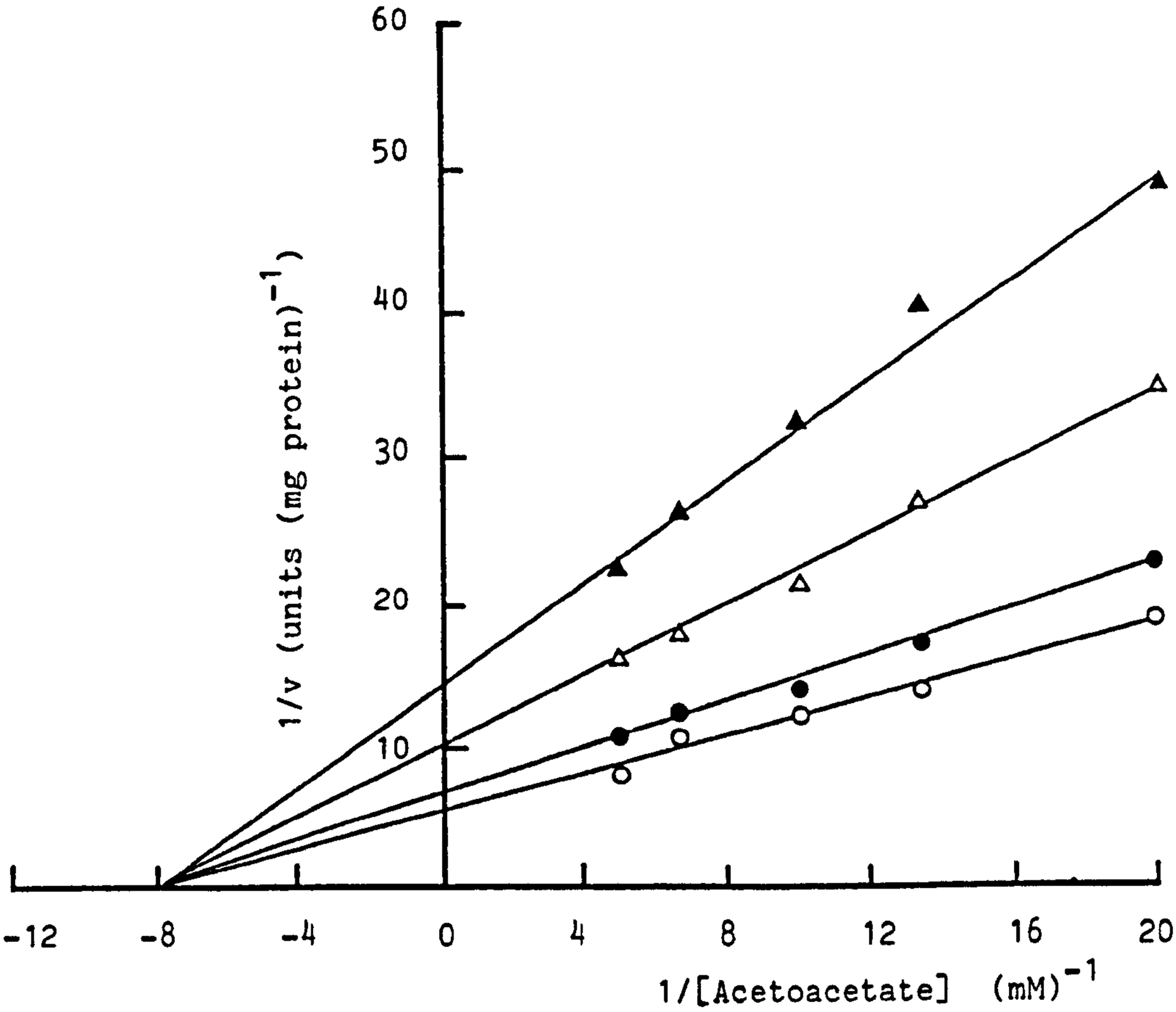


Figure 3.9 Lineweaver-Burk Transformation of Kinetic Data for the Reduction of Acetoacetate, Catalysed by 3-HBD at Different Initial Levels of NADH

Cuvettes contained in a total reaction volume of 3 ml: Tris-HCl buffer (100 mM, pH 7.3); acetoacetate (0.05 - 0.2 mM); purified enzyme solution (30 ng of protein). Concentration of NADH (mM):

(○) 0.08; (●) 0.04; (△) 0.02; (▲) 0.01.

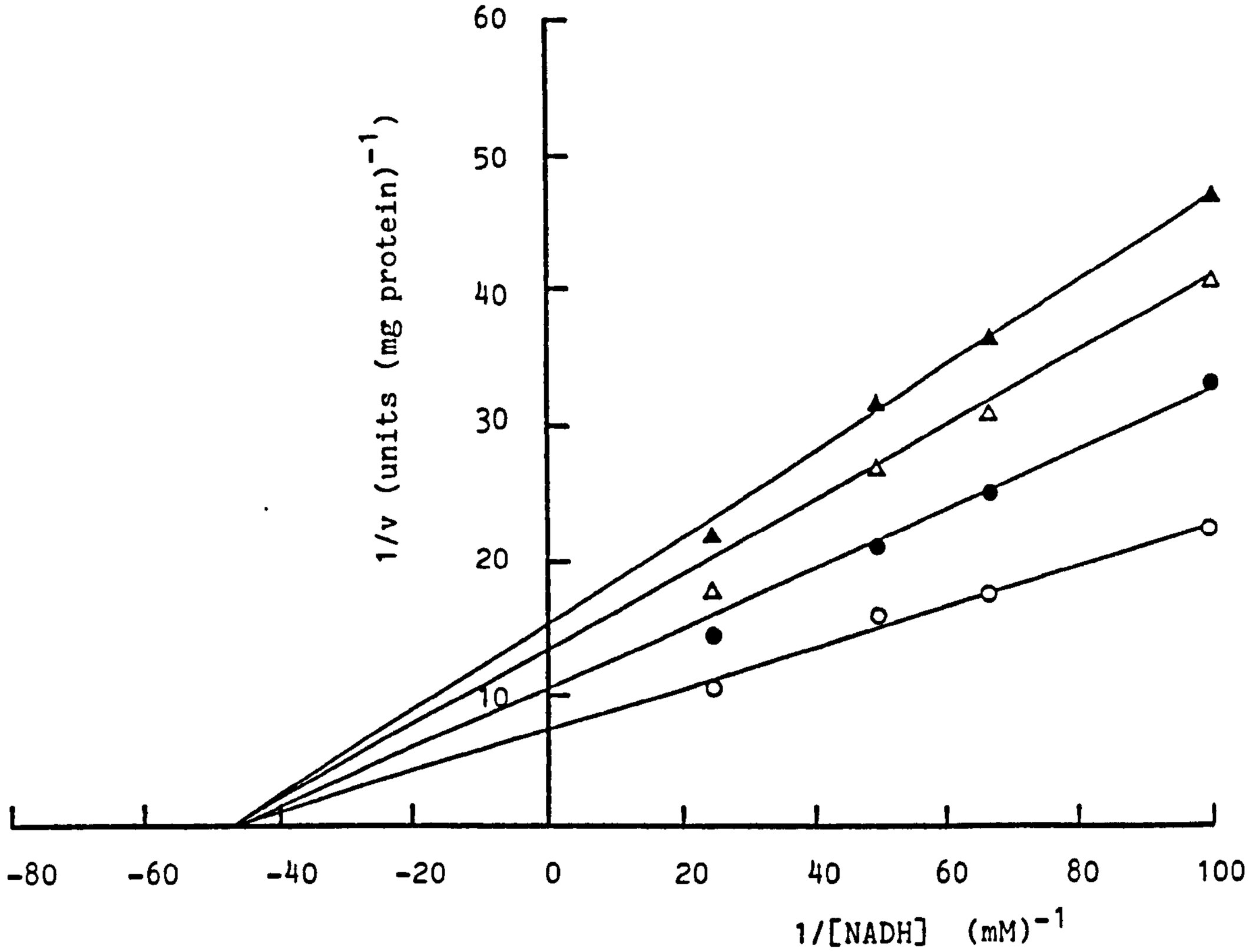


Figure 3.10 Lineweaver-Burk Transformaton of Kinetic Data for the Oxidation of NADH, Catalysed by 3-HBD at Different Initial Levels of Acetoacetate

Cuvettes contained in a total reaction volume of 3 ml: Tris-HCl buffer (100 mM, pH 7.3); NADH (3 mM) (0.01 - 0.08 mM); purified enzyme solution (30 ng of protein). Concentration of acetoacetate (mM): (○) 0.2; (●) 0.1; (△) 0.075; (▲) 0.05.

(acetoacetate and NAD) and product inhibition (D(-)-3-hydroxybutyrate and NAD). The significance of this data is highlighted by Cleland (1967) who suggested that reversible enzymes, which are inhibited by both the substrates and products of the reaction catalysed, are kinetically 'designed' to operate in the reverse direction to that measured (in this case, the oxidation reaction).

3.3.6 KINETIC ANALYSIS OF NADH AND ACETOACETATE INHIBITION OF THE OXIDATION REACTION CATALYSED BY 3-HBD

Kinetic analysis of both NADH and acetoacetate inhibition of the oxidation reaction was performed by varying the concentration of each substrate (D(-)-3-hydroxybutyrate and NAD) in turn, at various fixed levels of the inhibitor; the concentration of the second substrate was held constant and initial velocities measured. The double reciprical plots (Lineweaver & Burk, 1934) obtained at the various levels of inhibitor were examined to see whether the presence of the inhibitor increased the slope, the vertical intercept, or both, corresponding to competitive, uncompetitive or non-competitive inhibition. Furthermore, by replotting slopes and/or intercepts versus inhibitor concentration, the inhibition constant for the slope (K_{is}) or intercept (K_i) can be derived. By combination of these data it was possible to determine the relative potency of each inhibitor for the enzyme and also to predict the point at which it interacts with the enzyme reaction sequence (Cleland, 1967; 1970). Consequently, as NADH and acetoacetate are also products of the oxidation reaction, information on the reaction mechanism could also be infered from the results of this study.

A summary of NADH and acetoacetate inhibition patterns with respect to variable D(-)-3-hydroxybutyrate and NAD concentrations, together with the associated inhibitor constants, is presented in Table 3.3.

By increasing the level of NADH at fixed (saturating) D(-)-3-hydroxybutyrate and variable NAD concentration, an increase in the slope of the double reciprical plot revealed a competitive inhibition of 3-HBD by NADH with respect to NAD concentration (Figure 3.11.1). A succession of intersecting lines on the horizontal axis is indicative of this type of interaction and predicts that both cofactors are capable of binding to the same enzyme form. Secondary plot data of slope values versus inhibitor (NADH) concentration indicates that NADH inhibits oxidation in a linear fashion with respect to NAD concentration (Figure 3.11.2) and suggested that NADH interacts at only one point in the reaction mechanism of the enzyme (Cleland, 1967). Consequently, <u>in vivo</u> inhibition of 3-HBD by NADH has the effect of reducing the 'pool' of this enzyme available for the oxidation of D(-)-3-hydroxybutyrate and a K_i value of 35 μM highlights the efficiency of this compound as an inhibitor of the enzyme.

With NAD as a variable substrate, at a fixed non-saturating concentration of D(-)-3-hydroxybutyrate, an increase in the level of acetoacetate had the effect of increasing both the slope and intercept of the double reciprical plot (Figure 3.12.1). This kinetic pattern is characteristic of a non-competitive inhibition of the enzyme and predicts that the inhibitor and the variable substrate (NAD) combine

Inhibitor	Variable Substrate (range, mM)	Fixed Substrate (mM)	Inhibition Pattern*	K_{ii}+ (mM)	K_{is}+ (mM)
NADH	NAD (0.05 - 0.6)	D-3-HBA (10.0, saturating)	C	-	0.035
Acetoacetate	NAD (0.05 - 0.6)	D-3-HBA (0.2, non-saturating)	NC	0.53	0.56
Acetoacetate	D-3-HBA (0.5 - 2.0)	NAD (0.1, non-saturating)	NC	0.54	0.54

Abbreviations: D-3-HBA, D(-)-3-hydroxybutyrate

Table 3.3 Product Inhibition Patterns for the Oxidation of D(-)-3-Hydroxybutyrate by 3-HBD

* C and NC denotes competitive and non-competitive inhibition respectively.

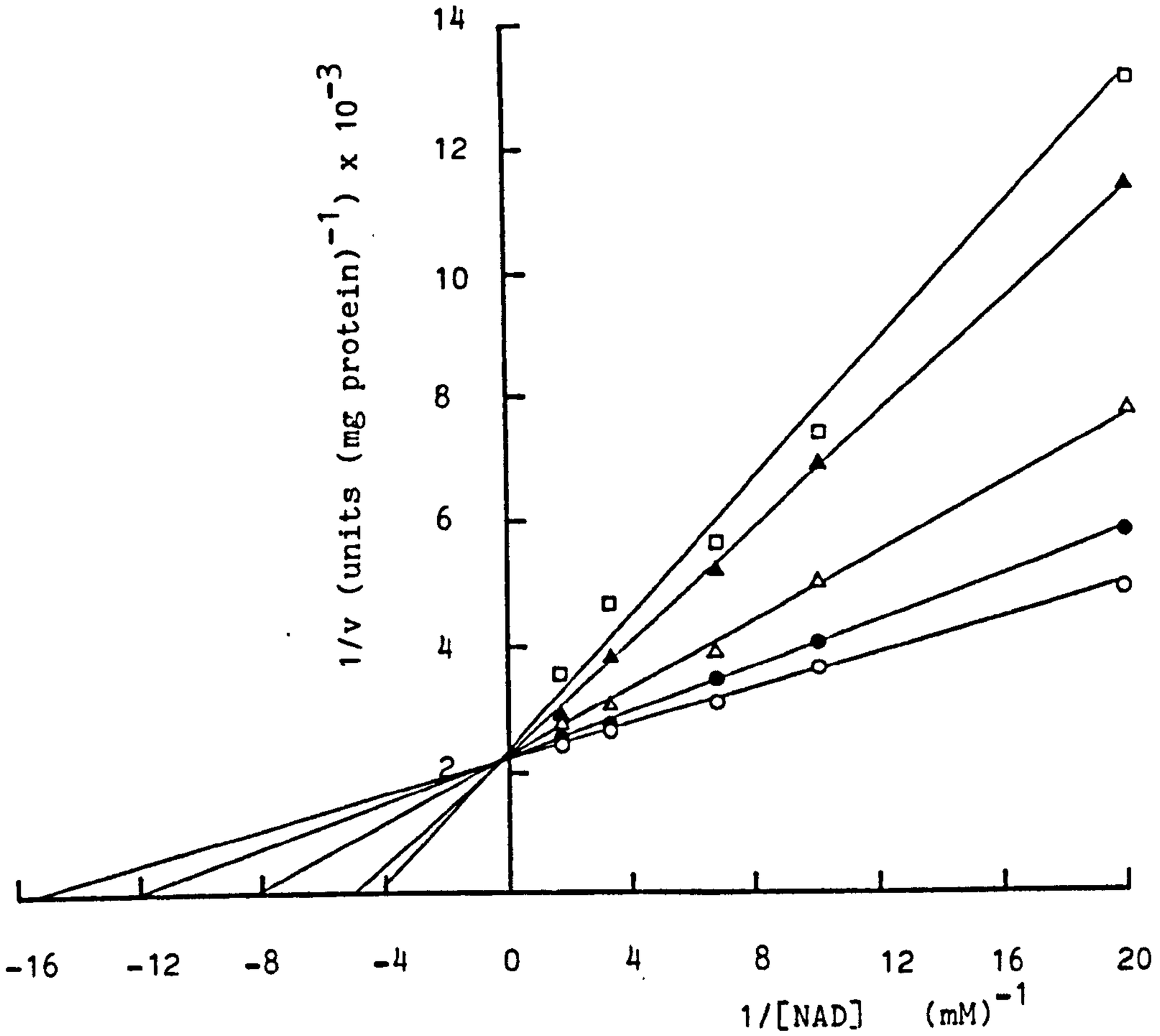
+ K_{is} and K_{ii} represents inhibition constants from slope and intercept plots, respectively.

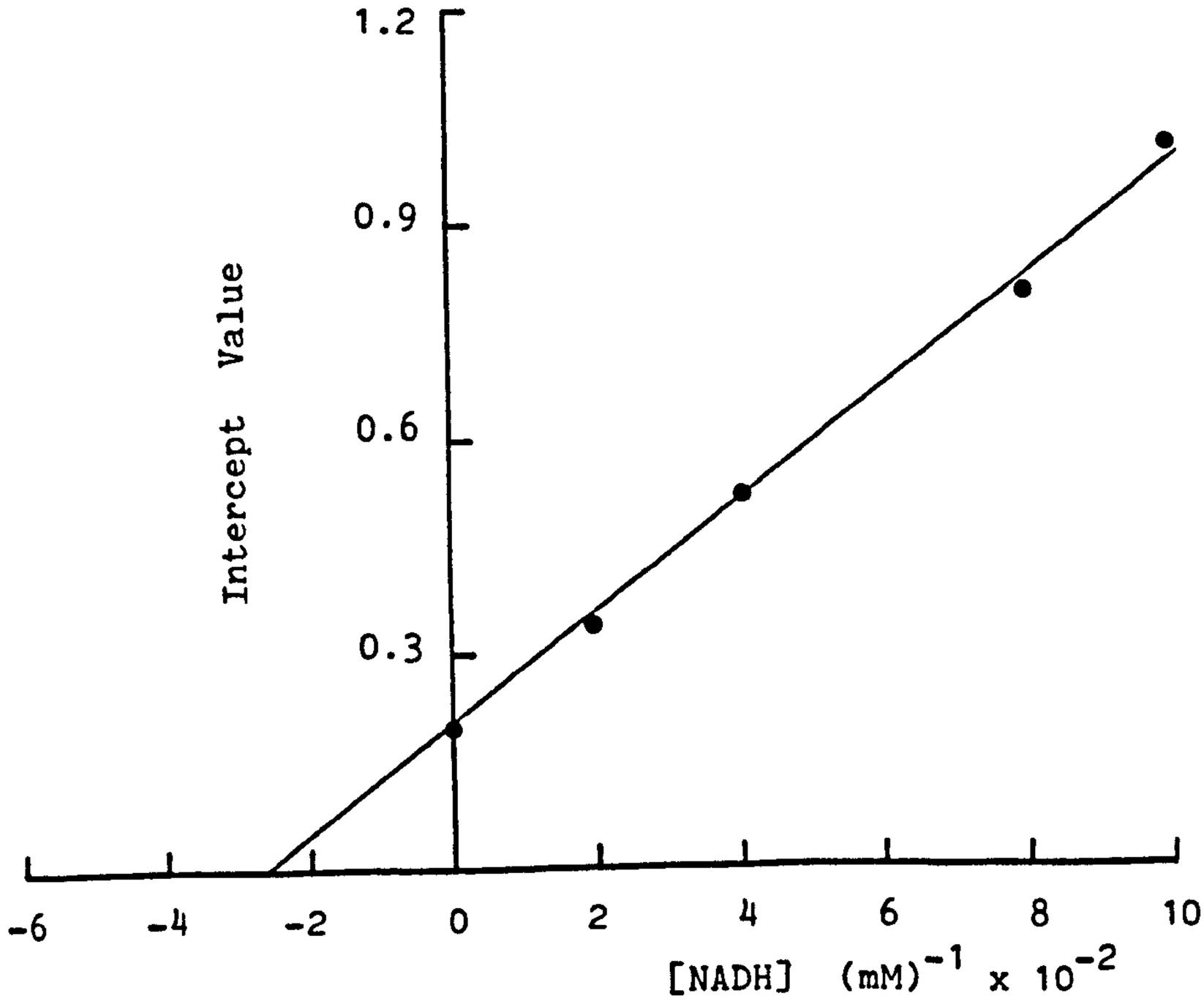
Figure 3.11 The Effect of NADH Concentration on the Initial Velocity of the Oxidation Reaction, Catalysed by 3-HBD in the Presence of Fixed, Saturating D(-)-3-Hydroxybutyrate and Variable NAD Concentration

Cuvettes contained in a total reaction volume of 3 ml: Tris-HCl buffer (100 mM, pH 8.2); D(-)-3-hydroxybutyrate (10 mM); NAD (0.05 - 0.6 mM); purified enzyme solution (30 ng of protein). Concentration of NADH (mM): (○) 0; (●) 0.02; (△) 0.04; (▲) 0.08; (□) 0.1.

(1) Lineweaver-Burk transformation of data.

(2) Secondary re-plot of slope values obtained from (1).


14
12
10
8
6
4
2
1/v (units (mg protein)$^{-1}$) x 10^{-3}
-16
-12
-8
-4
0
4
8
12
16
20
1/[NAD] (mM)$^{-1}$


1.2
0.9
0.6
0.3
Intercept Value
-6
-4
-2
0
2
4
6
8
10
[NADH] (mM)$^{-1}$ x 10^{-2}

with different enzyme forms (Cleland, 1967). Similarly with D(-)-3-hydroxybutyrate as the variable substrate (non saturating NAD), a non-competitive inhibition pattern was obtained (Figure 3.13.1), predicting that acetoacetate combines to a different enzyme form than the variable substrate, D-(-)-3-hydroxybutyrate. In both cases indicated above, different levels of inhibitor lead to a family of lines fanning out from a common point of intersection ($-1/K_m$) on the abscissa, suggesting that the inhibitor constants for the slope and intercept are equal. This is confirmed by secondary replots of slope and intercept values versus inhibitor (acetoacetate) concentration; these also show a linear relationship in each case (Figures 3.12.2; 3.13.2 and 3.13.3). The K_{ii} and K_{is} values are similar for each separate study and it can be predicted that acetoacetate combines at only one point in the reaction sequence (Cleland, 1967). Furthermore, the high K_{ii} and K_{is} obtained are indicative of the insensitivity of 3-HBD to inhibition by acetoacetate.

On inspection, the kinetic data appears to be consistent with an ordered Bi-Bi mechanism such as that shown by other 3-HBD's (Hurst et al., 1973; Klugger et al., 1978) and many pyridine nucleotide-linked dehydrogenases (Tan et al., 1975; Dalziel, 1975). Cleland (1963) has provided a number of rules for predicting dead end or product inhibition patterns without deriving the corresponding rate equation. For such an ordered Bi-Bi reaction mechanism where NAD preceeds D(-)-3-hydroxybutyrate addition to the enzyme and the products (acetoacetate and NADH) are released in an ordered fashion, he predicted a pattern identical to that achieved in this study.

Figure 3.12 The Effect of Acetoacetate Concentration on the Initial Velocity of the Oxidation Reaction, Catalysed by 3-HBD in the Presence of Fixed, Non-Saturating D(-)-3-Hydroxybutyrate and Variable NAD Concentration.

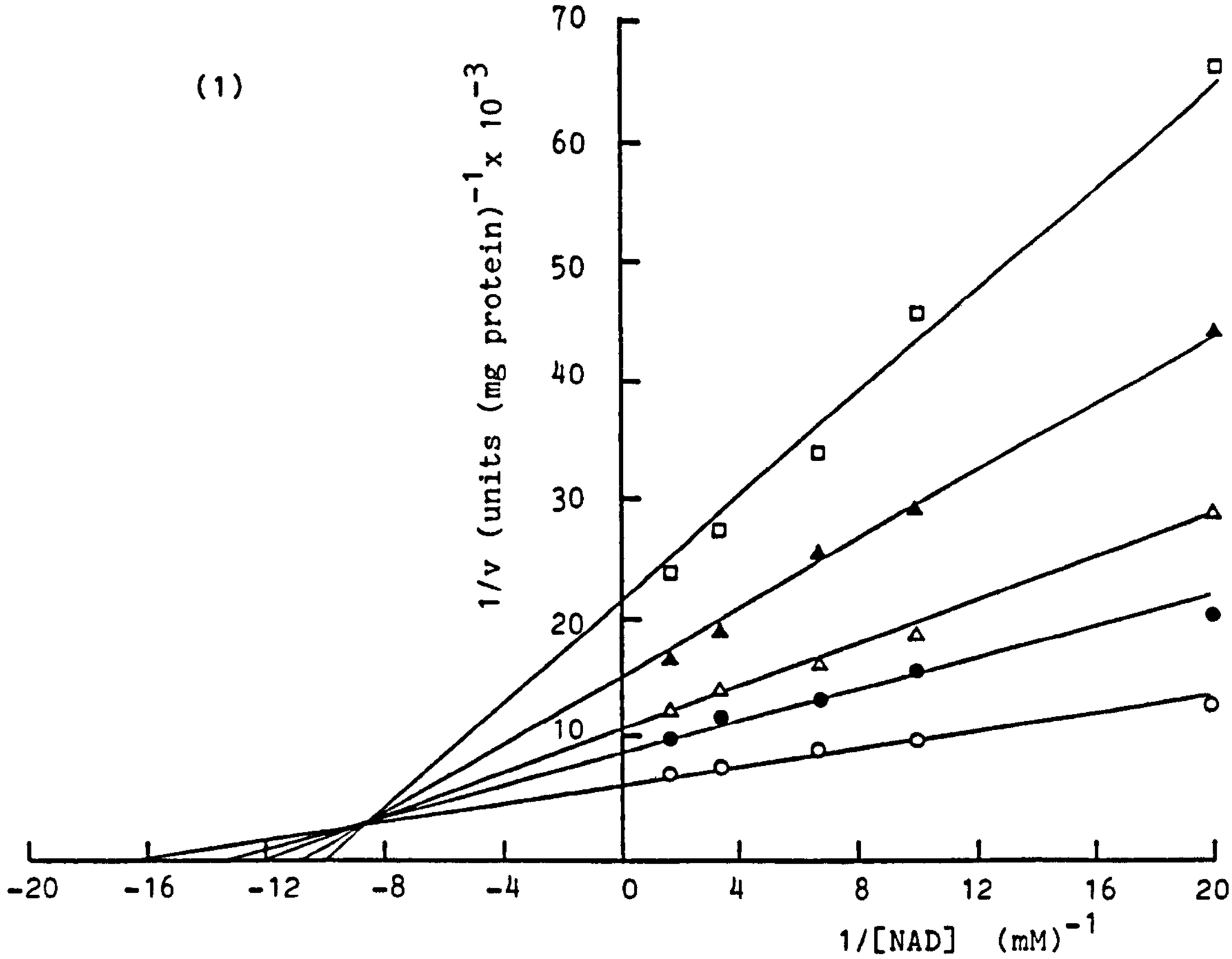
Cuvettes contained in a total reaction volume of 3 ml: Tris-HCl buffer (100 mM, pH 8.2); D(-)-3-hydroxybutyrate (0.2 mM); NAD (0.05 - 0.6 mM); purified enzyme solution (30 ng of protein). Concentration of acetoacetate (mM): (○) 0; (●) 0.2; (△) 0.4; (▲) 0.8; (□) 1.2.

(1) Lineweaver-Burk transformation of data.

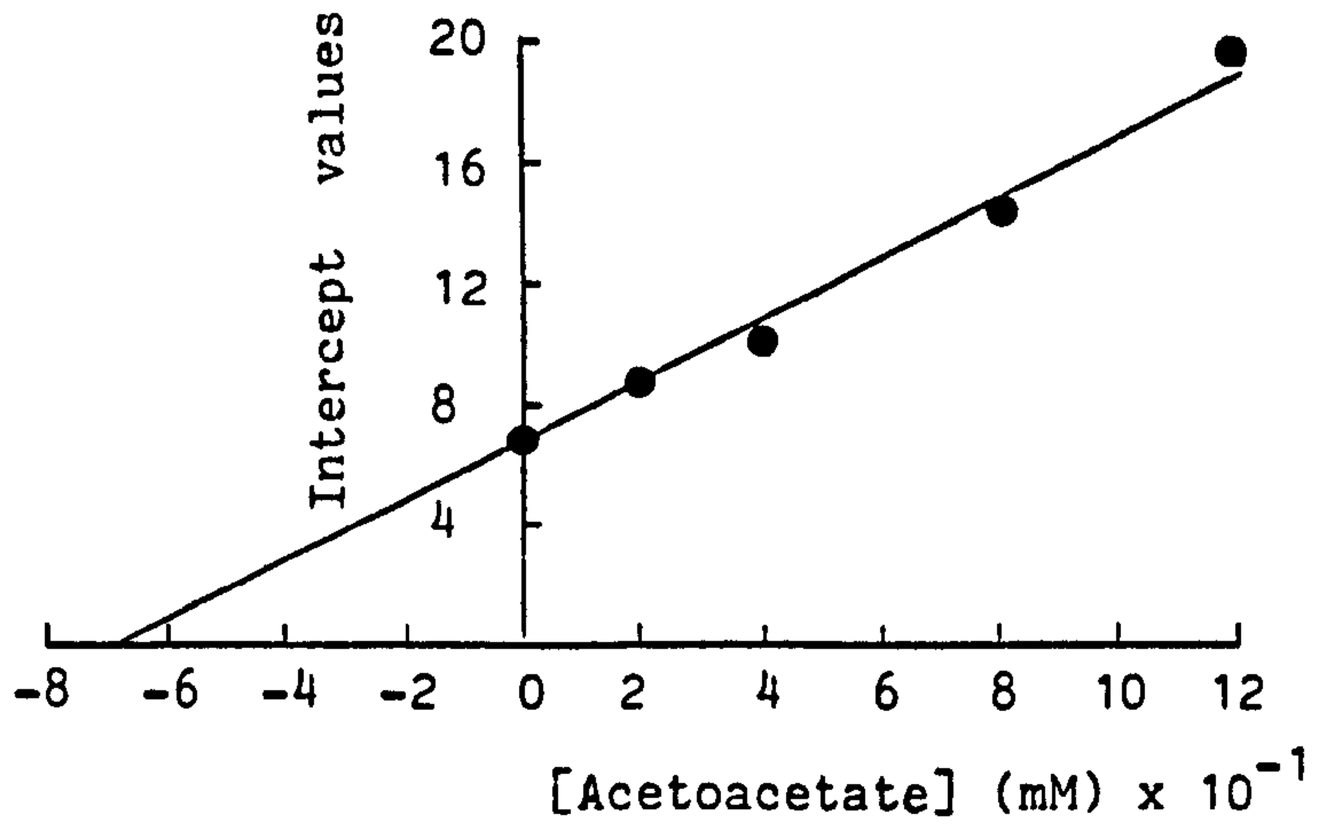
(2) Secondary re-plot of slope values obtained from (1).

(3) Secondary re-plot of intercept values obtained from (1).

(1)


1/v (units (mg protein)$^{-1}$ x 10^{-3}
70
60
50
40
30
20
10
-20
-16
-12
-8
-4
0
4
8
12
16
20
1/[NAD] (mM)$^{-1}$

(2)


Intercept values
20
16
12
8
4
-8
-6
-4
-2
0
2
4
6
8
10
12
[Acetoacetate] (mM) x 10^{-1}

(3)

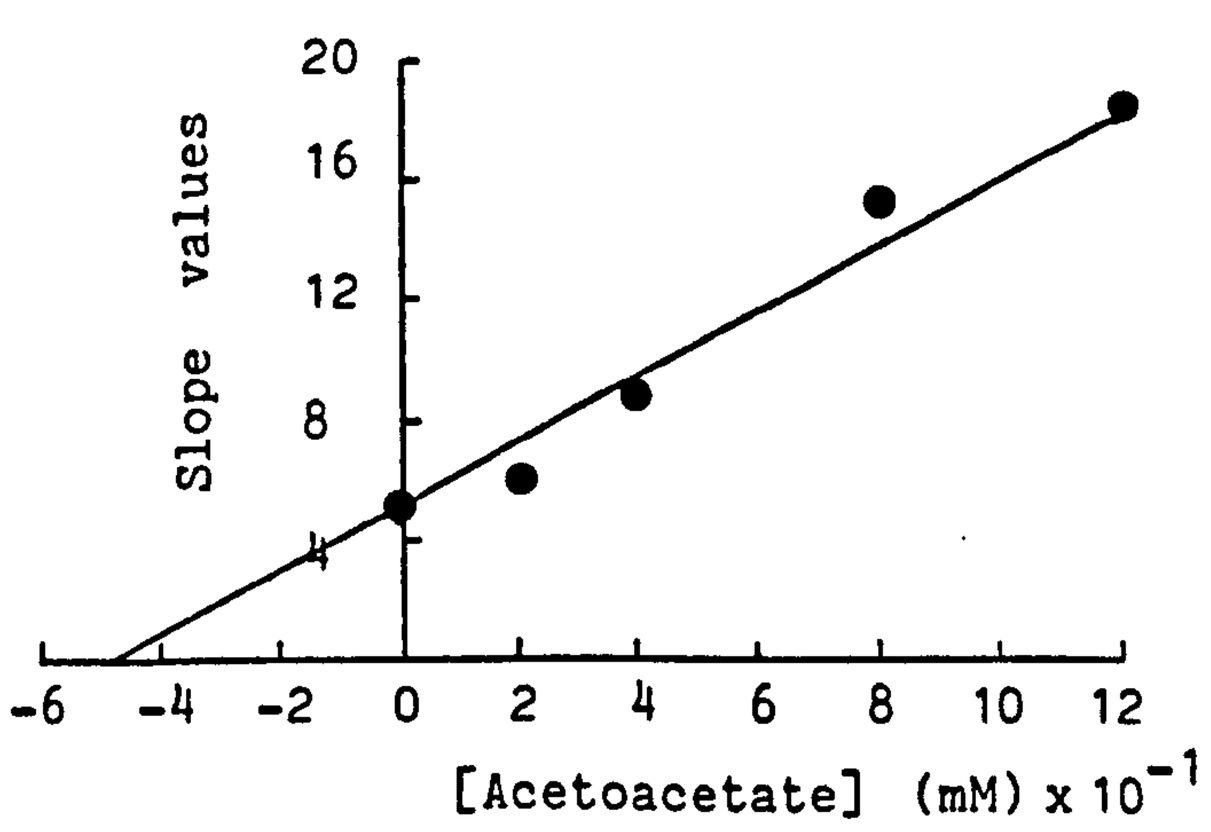

Slope values
20
16
12
8
4
-6
-4
-2
0
2
4
6
8
10
12
[Acetoacetate] (mM) x 10^{-1}

Figure 3.13 The Effect of Acetoacetate Concentration on the Initial Velocity of the Oxidation Reaction, Catalysed by 3-HBD in the Presence of Fixed Non-Saturating NAD and Variable D(-)-3-Hydroxybutyrate Concentration

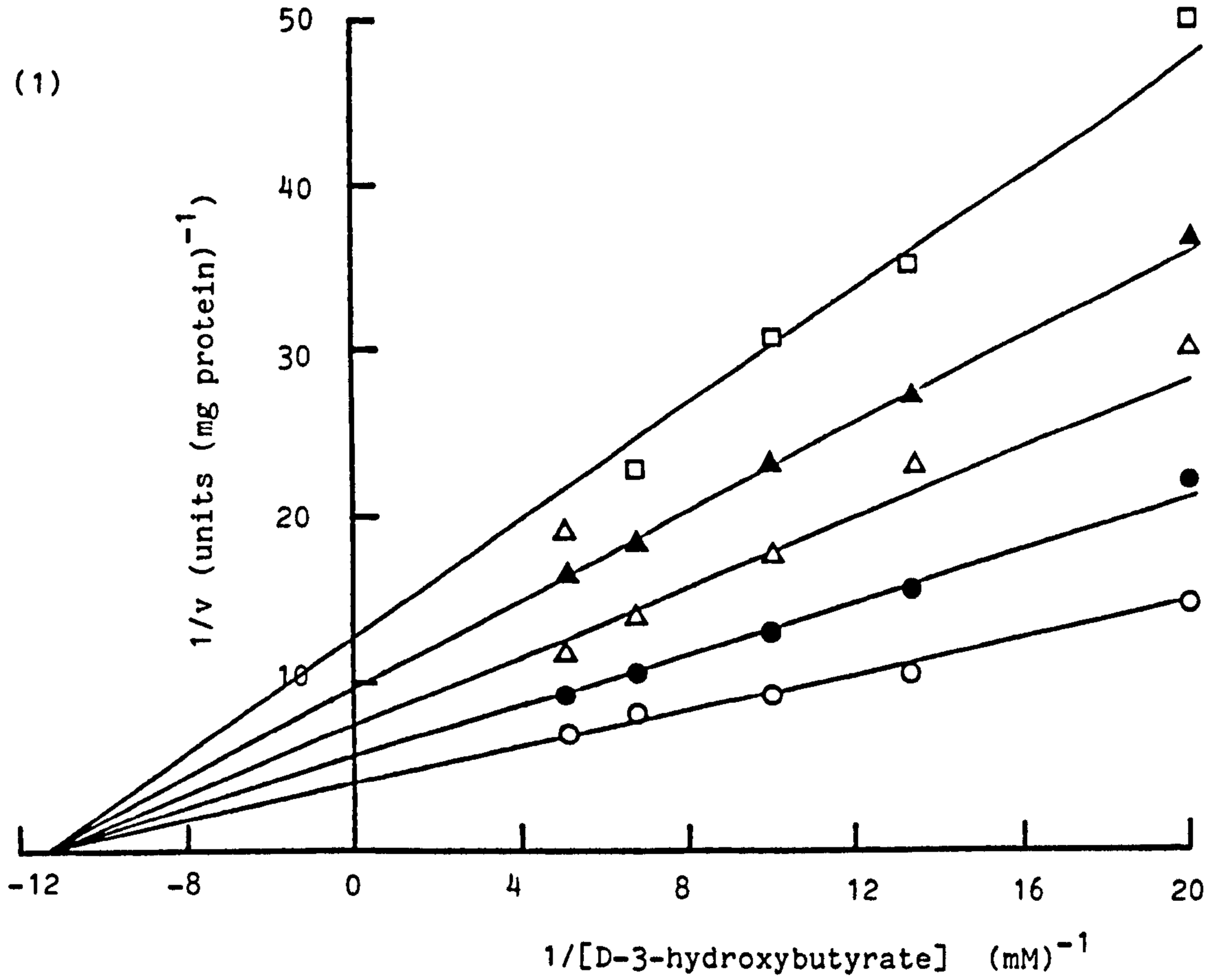
Cuvettes contained in a reaction volume of 3 ml: Tris-HCl buffer (100 mM, pH 8.2); NAD (0.1 mM); D(-)-3-hydroxybutyrate (0.5 - 2.0 mM); purified enzyme solution (30 ng of protein). Concentration of acetoacetate (mM): (○) 0; (●) 0.2; (△) 0.4; (▲) 0.8; (□) 1.2.

(1) Lineweaver-Burk transformation of data.

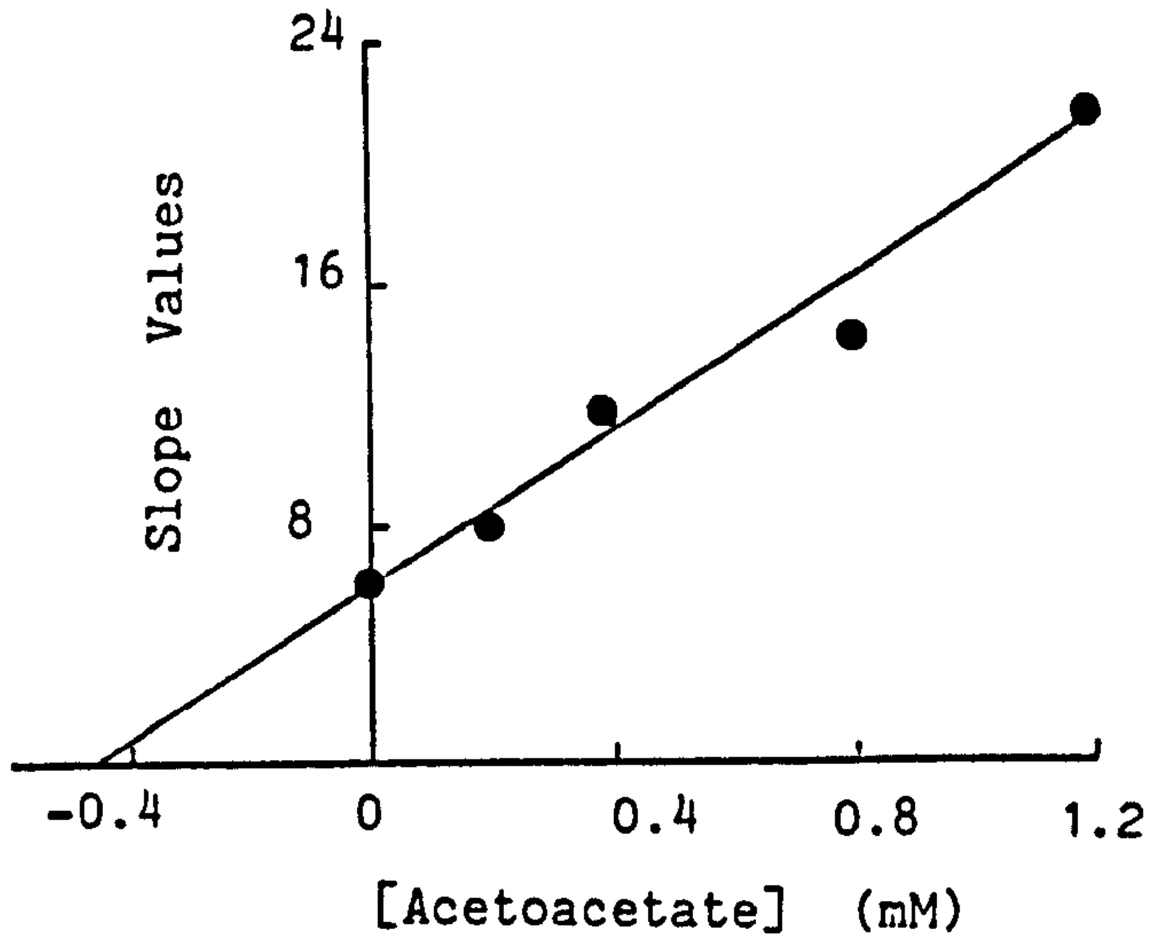
(2) Secondary re-plot of slope values obtained from (1).

(3) Secondary plot of intercept values obtained from (1).

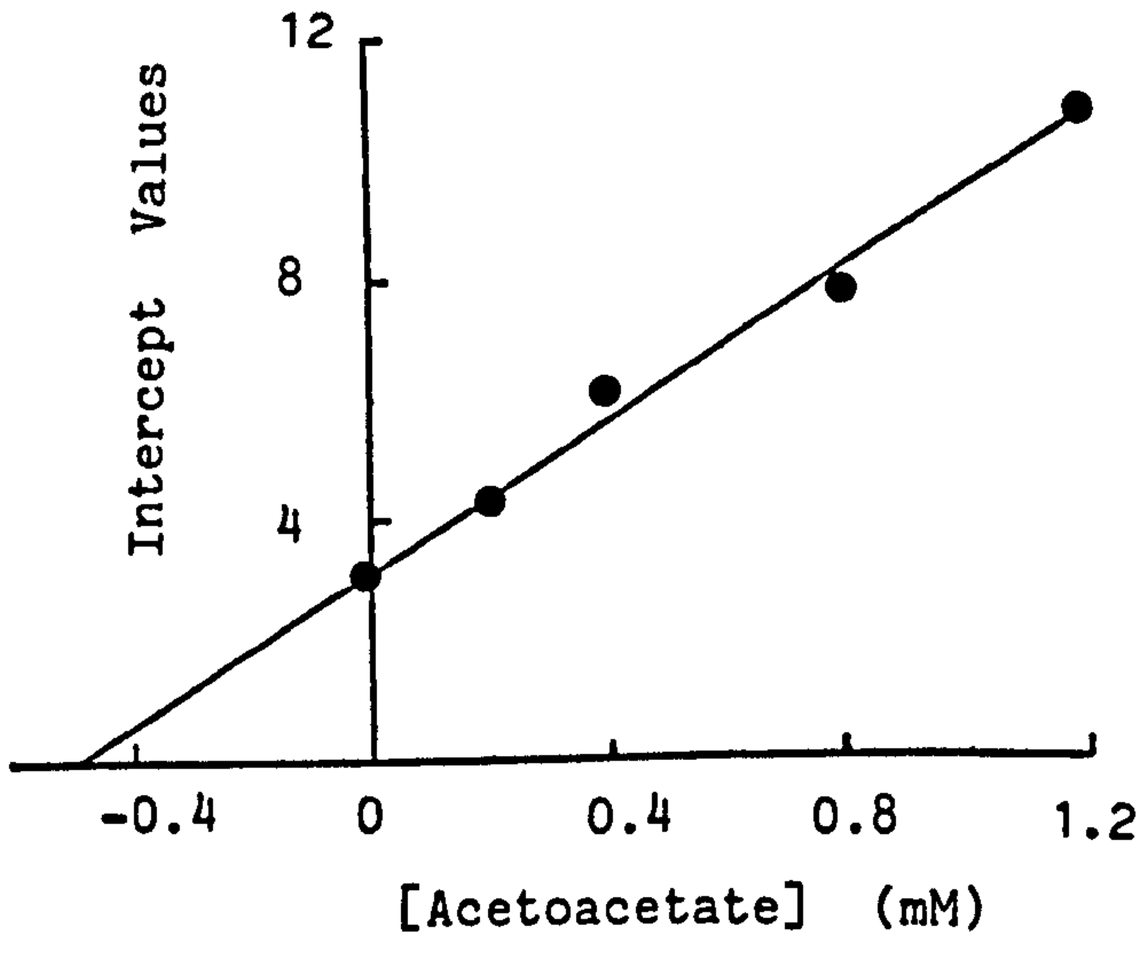
(1)


50
40
30
20
10
1/v (units (mg protein)$^{-1}$)
-12
-8
0
4
8
12
16
20
1/[D-3-hydroxybutyrate] (mM)$^{-1}$

(2)


24
16
8
Slope Values
-0.4
0
0.4
0.8
1.2
[Acetoacetate] (mM)

(3)


12
8
4
Intercept Values
-0.4
0
0.4
0.8
1.2
[Acetoacetate] (mM)

Consequently the kinetic patterns obtained have the following implications:

(1) The only reactants which combine with the same enzyme form are NAD and NADH. Therefore, competitive inhibition is observed; NAD is bound first by the free enzyme and NADH released last in the reaction sequence (see equation 3.2).

(2) Acetoacetate combines with a different enzyme form than either NAD or D(-)-3-hydroxybutyrate. This is consistent with this product preventing dissociation of NADH from the enzyme complex (equation 3.2). Its presence reestablishes the reversability of the E.NADH.Acetoacetate to E.NADH step, and the reversal of this step effectively displaces the equilibrium of the reaction in the direction of D(-)-3-hydroxybutyrate formation by opposing the forward reaction.

Unlike lactate dehydrogenase, where the product, pyruvate, acts as a product inhibitor and a dead end inhibitor, forming the abortive complex E.pyruvate.NAD, acetoacetate is exclusively a product inhibitor of 3-HBD. Essentially, acetoacetate is unable to bind to the enzyme form, which has previously bound NAD, to form a dead end complex. If this was the case a competitive inhibition pattern would be expected when D(-)-3-hydroxybutyrate is varied with respect to increasing concentration of acetoacetate.

3.3.7 EQUILIBRIUM CONSTANT OF 3-HBD

The equilibrium of the reaction:

$$\text{D(-)-3-Hydroxybutyrate} + \text{NAD} \rightleftharpoons \text{Acetoacetate} + \text{NADH} + \text{H}^+$$

was approached from the left hand side over the range pH 7.5 - 8.2 (Table 3.6). The equilibrium constant, K, was determined from the equation:

$$K = \frac{[\text{ACETOACETATE}]\ [\text{NADH}]\ [\text{H}^+]}{[\text{D(-)-3-HYDROXYBUTYRATE}]\ [\text{NAD}]}$$

K was determined to be 6.65×10^{-10} at pH 7.5, indicating that the equilibrium of the reaction does not favour the formation of acetoacetate from D(-)-3-hydroxybutyrate. Buffering at a higher pH effectively shifts the equilibrium in favour of acetoacetate formation by trapping the proton produced in the reaction. Subsequently, the reaction becomes more 'favourable' (or less unfavourable) with increasing pH.

pH	H^+ (μM)	D-3HBA (μM)	NAD^+ (μM)	AC (μM)	NADH (μM)	10^{-10} K_{eq} (M)
7.5	0.0316	970.88	64.40	35.6	35.6	6.65
7.8	0.0158	955.60	55.60	44.40	44.40	5.86
8.2	0.0063	941.76	41.76	58.24	58.24	5.43

Abbreviations: D-3-HBA, D(-)-3-hydroxybutyrate; AC, acetoacetate.

Table 3.4 Equilibrium Constant of 3-HBD from M. trichosporium OB3b

The reaction mixture contained (final concentration) Tris-HCl buffer (100 mM, pH 7.5 to 8.2); D(-)-3-hydroxybutyrate (1 mM); NAD (0.3 mM) and purified enzyme solution (3 μg of protein) in a total reaction volume of 3 ml. The reaction was started by the addition of D(-)-3-hydroxybutyrate and the extinction recorded continuously until equilibrium was reached. Final concentrations of each component at equilibrium were calculated from the change in extinction at 340 nm.

3.4 DISCUSSION

The method established for the purification of 3-HBD from M. trichosporium OB3b is a good example of the application of triazine dye affinity chromatography in the purification of nucleotide-dependent enzymes. Although triazine dye affinity chromatography is probably more correctly described as "pseudo affinity chromatography", in that a large number of diverse proteins can be bound through the dye ligand, this study has highlighted the pertinent points required to introduce selectivity in resolving the enzyme from the crude extract. The versatility of these affinity matrices in purifying a wide range of enzymes is highlighted by the differential desorption of 3-HBD from the same matrix by both ionic and cofactor elution. Simply by using this one chromatographic technique the enzyme was separated from "contaminating protein" on the basis of affinity for the dye ligand, the difference in binding affinities for protein species and their relative cofactor specificity.

The high yield of homogeneous enzyme, which was in excess of 70 %, compares favourably with the recovery of 3-HBD from R. spheroides where triazine dye affinity chromatography was also used in the purification procedure (Scawen et al., 1982). However, this result contrasts with the low yield of 3-HBD from other bacterial sources of the enzyme, which employed conventional chromatographic techniques in the purification protocol (Bergmeyer et al., 1967; Dhariwal & Venkitasubramanian, 1978; Nakada et al., 1981; Matyskova et al.,

1985). Furthermore, the specific activity of the purified enzyme is the highest recorded for 3-HBD from any source, and this too reflects the efficiency of the purification scheme developed in this study.

To a large extent, the enzymes isolated to date, including the methanotrophic enzyme, share a number of closely related properties (Table 3.7). The pH optima for the oxidation reaction exhibit a similar range in all cases and there is an absolute dependence upon NAD as the cofactor; NADP is unable to replace NAD in the oxidation of D(-)-3-hydroxybutyrate. Furthermore, K_m values for D(-)-3-hydroxybutyrate and NAD are of the same order.

Indirect evidence for the involvement of a thiol group(s) in the catalytic mechanism of the methanotrophic enzyme can be inferred from stability studies. Sulphydryl reagents effect stabilisation of enzyme activity together with both NAD and NADH. This correlates with a role assigned to thiol groups in dehydrogenase enzymes, which include the binding of nucleotides to proteins (Bergmeyer et al., 1967).

In contrast to the 3-HBD from R. rubrum (Shuster & Doudoroff, 1962) the enzyme from M. trichosporium OB3b was not cold labile. The methanotrophic enzyme was insensitive to metal ions, both in terms of their role as stabilising agents and as activating agents. Furthermore, the enzyme is uniquely monomeric, and its regulation significantly different to other bacterial sources of the enzyme, where its role in the control of PHB mobilisation has been established (Senior & Dawes, 1973; Oeding & Schlegel, 1973; Nakada et al., 1981).

Bacterium	Zoogloea[1] ramigera	Rhodopseudomonas[2] spheroides	Azotobacter[3] beijerinkii	Pseudomonas[4] lemoignei	Rhodospirillum[5] rubrum	Methylosinus[6] trichosporium OB3b
Molecular Weight	112,000	85,000	-	-	-	26,000
Michaelis Constants (M x 10^{-3})						
D(-)-3-hydroxybutyrate	0.32	0.41	0.877	0.6	0.84	1.33
NAD	0.057	0.08	0.07	-	0.07	0.062
Acetoacetate	0.15	0.28	-	0.2	0.07	0.13
NADH	0.015	0.054	-	-	-	0.34
pH optima (oxidation reaction)	8.0	8.0-9.2	8.4-8.5	8.0	8.0	8.2
Inhibitors	Acetyl-CoA, NADH, D-lactate, Acetoacetate	Succinate, D-lactate, 2-Hydroxybutyrate	Pyruvate, 2-Oxoglutarate, NADH	D-lactate	-	NADH Acetoacetate
Stabilising Agents	Sulphydryl Compounds	NADH, NAD, Ca^{2+}	-	Mg^{2+}, Ca^{2+}, NAD		NADH, NAD, Sulphydryl Compounds

Table 3.7 3-HBD from Various Bacterial Sources

1 Nakada et al. (1981); 2 Bergmeyer et al. (1967); 3 Senior & Dawes, (1973); 4 Delafield et al. (1965); 5 Shuster & Doudoroff, (1962); 6 This thesis.

Throughout the course of any biochemical pathway the point at which metabolic regulation is exerted should ideally occur early in the pathway and shortly after branch points in order to avoid long stretches of uncontrolled metabolism (Rolleston, 1972). With this in mind, the control of PHB mobilisation would ideally occur on the PHB granule, at the level of polymer hydrolysis. This would provide the most energetically inexpensive point to effect metabolic control over the pathway. However, the enzyme/PHB granule system has been shown to be extremely complex (Merrick & Doudoroff, 1964; Griebel & Merrick, 1971), and the control mechanism of polymer mobilisation remains largely unresolved.

In the absence of data on the control of polymer hydrolysis, 3-HBD has been implicated as a key regulatory enzyme in the control of PHB mobilisation (Dawes & Senior, 1973). Clearly, as PHB serves both as a carbon and energy source, careful control of D(-)-3-hydroxybutyrate oxidation is necessary in order to maintain the energetic requirements of the organism, via the NADH pool, and the supply of carbon precursors through the activation of acetoacetate. By this reasoning, 3-HBD fulfills an important metabolic role on the pathway of PHB mobilisation. The same reasoning has been applied to the control of PHB mobilisation in M. trichosporium OB3b.

Product inhibition studies have shown that the methanotrophic enzyme is highly susceptible to inhibition by NADH, as indicated by a K_{is} of 35 μM for this metabolite. This suggests that the activity of the enzyme in vivo is extremely sensitive to fluctuations in the redox state (NADH/NAD couple) of the cell. If the regulation of 3-HBD

was the major control point of PHB mobilisation then one might expect that the ratio of NADH/NAD would be high during PHB synthesis and low during its mobilisation. In this way, the metabolism of PHB would be dependent upon the energetic requirements of the cell, which in turn relates to the level of carbon available for oxidative purposes.

Although the methanotrophic enzyme is also susceptible to non-competitive inhibition by acetoacetate, the importance of this metabolite in the control of 3-HBD activity is probably limited in comparison to that of NADH. A major point of interest, however, is the absence of metabolic inhibition of the enzyme other than products of the oxidation reaction. These results deviate from the trend exhibited by all the 3-HBD's isolated to date, which have been shown to possess a number of competitive inhibitors of this reaction. Of interest in this study are the 3-HBD's which have been studied with regard to evaluating their role in the regulation of PHB mobilisation. The enzymes from A. beijerinkii and A. eutrophus H16 are both inhibited by pyruvate and additionally by 2-oxoglutarate and oxaloacetate respectively. The enzyme from Z. ramigera I-16-M is competitively inhibited by lactate and acetyl-CoA. Competitive inhibition of the oxidation reaction is thought to reflect an absence in steric constriction of the enzyme to closely related metabolites (Klugger et al., 1978). In contrast, the active site of the methanotrophic enzyme, on binding NAD, can differentiate between its substrate and a large number of closely related compounds.

The significance of the regulatory data indicated above is that PHB mobilisation, in non-methanotrophs, appears to be regulated

through feedback inhibition upon 3-HBD, by both the redox state of the cell and intermediates of carbon metabolism, either through the activity of the TCA cycle or through glucose catabolism. This type of regulation fits the role of PHB, as both a carbon and energy source. If 3-HBD can be visualised as a diagnostic enzyme of the role of the storage polymer in the respective organism, as citrate synthase is for the TCA cycle (Weitzman, 1980), then the results of this study tend to suggest that PHB is utilised primarily for energetic purposes in this methanotroph. This possibility is substantiated by studies on acetone accumulation in this organism, where cells incubated with an oxidisable substrate, like propene, produce acetone concomitant with PHB mobilisation (Thompson et al., 1976; Best, 1982). Therefore, under conditions where the cell requires reducing power for oxidative purposes, the NADH/NAD couple might be expected to shift to a value less than unity. Inhibition of 3-HBD is relieved and acetoacetate decarboxylase, which is present in this organism (Thompson et al., 1976; Best, 1982), serves to displace the thermodynamically unfavourable equilibrium of the reaction ($K = 6.65 \times 10^{-10}$ M at pH 7.5) in favour of PHB mobilisation. The apparently wasteful excretion of acetone might serve to prevent further metabolism of acetoacetate which might prove energetically expensive. In this way acetone might be excreted to maintain NADH levels.

C H A P T E R F O U R

THE PURIFICATION AND PROPERTIES OF ACETETOACETYL-COA SYNTHETASE FROM M. TRICHOSPORIUM OB3B

4.1 INTRODUCTION

The first step in the utilisation of acetoacetate, by bacteria following its formation through the depolymerisation of PHB and its subsequent dehydrogenation via D(-)-3-hydroxybutyrate, is its activation to acetoacetyl-CoA. This may be effected by one of two different enzymes:

(1) Acetoacetate:succinate CoA-transferase (EC 2.8.3.5), which catalyses the reaction:

$$\text{Acetoacetate} + \text{Succinyl-CoA} \rightleftharpoons \text{Acetoacetyl-CoA} + \text{Succinate}$$

(2) Acetoacetyl-CoA synthetase, which catalyses the reaction:

$$\text{Acetoacetate} + \text{ATP} + \text{CoA} \rightleftharpoons \text{Acetoacetyl-CoA} + \text{AMP} + PP_i$$

Once formed, acetoacetyl-CoA is cleaved to produce acetyl-CoA in the reaction catalysed by beta-ketothiolase (chapter 5).

Although Stadtman (1953) initially demonstrated acetoacetate:succinate CoA-transferase activity in extracts of Clostridium kluyveri, it was not until much later that its role in the pathway of PHB mobilisation was highlighted (Senior and Dawes, 1973). During this study Senior and Dawes reported a 26-fold purification of acetoacetate:succinate CoA-transferase from A. beijerinkii. The enzyme displayed conventional Michaelis-Menten kinetics with respect to both

Of the two enzymes known to participate in the reactivation of acetoacetate, little information is available at present on the properties of acetoacetyl-CoA synthetase. The enzyme was reported to be present in various mammalian tissue, yeast and R. rubrum (Stern & Ochoa, 1951; Stern et al., 1953; Stern, 1971; Buckley & Williamson, 1973). However, to date, only one group have succeeded in purifying acetoacetyl-CoA synthetase, and demonstrating activity associated with a discrete enzyme (Fukui et al., 1982). The enzyme was purified from Z. ramigera I-16-M and had an absolute requirement for ATP, CoASH, a monovalent cation (K^+, Rb^+, Cs^+ or NH_4^+) and a divalent cation (Mg^{2+}, Mn^{2+}, Ca^{2+}or Ni^{2+}) for the activation of acetoacetate, yielding acetoacetyl-CoA, AMP and pyrophosphate in equimolar amounts. The enzyme has a native molecular weight of 72,000, with K_m values for acetoacetate, CoASH and ATP of 7.6×10^{-5} M, 3.3×10^{-5} M and 9.1×10^{-5} M respectively. No acetoacetate:succinate CoA-transferase activity was detected in this organism.

In view of the limited studies on acetoacetyl-CoA synthetase from microorganisms, its metabolic significance with respect to PHB mobilisation awaits further clarification. In contrast however, significant advances have been made in assessing the metabolic role of acetoacetyl-CoA synthetase from mammalian tissues. The enzyme appears to play an important role in lipogenesis. This is largely based on the measurement of enzyme activity, which is greatest in tissues and situations associated with high rates of lipogenesis: e.g., developing rat brain (Bressler, 1963; Yeh et al., 1977) and the liver (Benito & Williamson, 1978) of lactating rats. Moreover the acetoacetyl-CoA synthetase activities in brain, mammary gland and adipose tissue is

acetoacetyl-CoA (K_m = 2.8 x 10^{-5} M) and succinate (K_m = 4.0 x 10^{-3} M). Although the equilibrium constant of this reaction does not appear to lie in the direction of PHB mobilisation (maximum initial rates were 1% of those recorded for the reverse reaction), it was suggested that under conditions which stimulate PHB mobilisation (low NAD(P)H, high CoASH and thus low acetoacetyl-CoA) the reaction would proceed towards acetoacetyl-CoA formation.

Acetoacetate:succinate CoA-transferase activity has also been implicated durng PHB mobilisation in A. eutrophus H16 (Oeding & Schlegel, 1973) and the facultative methylotroph, Ps. AM1 (Taylor & Anthony, 1976). Transferase activity in Ps AM1 did not appear to be inducible and its activity remained constant during the growth of this organism on a number of different carbon sources. Furthermore, malate, acetate, propionate, glycollate or formate were not activated by transfer of CoASH from acetoacetyl-CoA during this reaction, indicating the specificity of this enzyme for succinate.

Acetoacetate:succinate CoA-transferase activity is also demonstrable in most metabolically active tissues (Blair, 1969; Tildon & Sevdalian, 1972; Fenselau & Wallis, 1974). The enzyme is intimately associated with the control of ketogenesis, and therefore performs a distinctly dissimiar role to its bacterial equivalent. In view of these differences no further information on the mammalian enzyme will be presented at this point. The reader is referred to an excellent review by Robinson and Williamson (1980) where a wider discussion on its role in ketone body metabolism is presented.

remarkably similar to the *in vivo* rate of acetoacetate incorporation into lipid (Buckley & Williamson, 1975; Patel & Owen, 1975; Robinson & Williamson, 1978; Soling *et al.*, 1970). Such information is not directly applicable to bacterial systems. Nevertheless, a comparison of the roles played by the acetoacetate:succinate CoA transferase and acetoacetyl-CoA synthetase in mammalian tissue might help to highlight the significance of each enzyme in the pathway of PHB mobilisation.

At present, there is no information avaialble on acetoacetate activation in methanotrophs. This chapter investigates the route of acetoacetate utilisation in *M. trichosporium* OB3b and assesses the role and the significance of the enzyme in PHB mobilisation. Furthermore, the significane of this enzyme, in the wider context of methanotrophic carbon metabolism, in the light of the conclusions drawn from the study on 3-HBD (section 3.3), is discussed.

4.2 EXPERIMENTAL

4.2.1 PROTOCOL FOR THE PURIFICATION OF ACETOACETYL-COA SYNTHETASE FROM M. TRICHOSPORIUM OB3B

Frozen cells (200 g) were thawed and resuspended in potassium phosphate buffer (20 mM, pH 6.0, 200 ml) at room temperature. The cell free extract was prepared as described previously (section 2.5), with the exception that the final pH was adjusted to 6.0. All purification procedures were carried out at 4°C. Enzyme activity was monitored by method A in the cell free extract but by method B for all subsequent steps.

STEP 1: Chromatography on DEAE-Sepharose

The cell free extract was applied to a column of DEAE-Sepharose (5 x 6.5 cm), preequilibrated with potassium phosphate buffer (20 mM, pH 6.0). The column was washed with equilibration buffer (800 ml) to remove unbound protein and the enzyme eluted with a linear gradient (800 ml) of potassium chloride (0 - 0.4 M) in the equilibration buffer at a flow rate of 60 ml. h^{-1}. Active fractions (8 ml) were pooled and dialysed by diafiltration against five volumes of potassium phosphate buffer (20 mM, pH 7.0).

STEP 2: Chromatography on Procion Red H-3B Sepharose

The dialysate from the previous step was applied to a column of Red H-3B Sepharose (2.5 x 14 cm) preequilibrated with potassium phosphate buffer (20 mM, pH 7.0). The column was washed with equilibration buffer (100 ml) and the enzyme eluted in an upwards direction, at a flow rate of of 30 $ml.h^{-1}$, using the same buffer containing AMP (5 mM, 25 ml). Following the application of AMP, the column was washed with with equilibration buffer (30 ml) until elution of the enzyme was complete. All enzyme activity was collected and concentrated under nitrogen by ultrafiltration (Amicon, 10,000 molecular weight cut-off membrane).

STEP 3: Chromatography on Sephacryl S-300

The active extract was applied to a column of Sephacryl S-300 (2.5 x 60 cm), preequilibrated with Tris-HCl buffer (50 mM, pH 7.5) at a flow rate of 30 ml. h^{-1}. The combined active fractions were concentrated as described above and used in subsequent analysis.

4.2.2 METHODS USED TO ASSAY ACETOACETYL-COA SYNTHETASE

Acetoacetyl-CoA synthetase activity was measured by one of four different methods: following the formation of (A) acetyl-CoA, (B) enolate, (C) NAD or (D) AMP. These are summarised schematically in Figure 4.1.

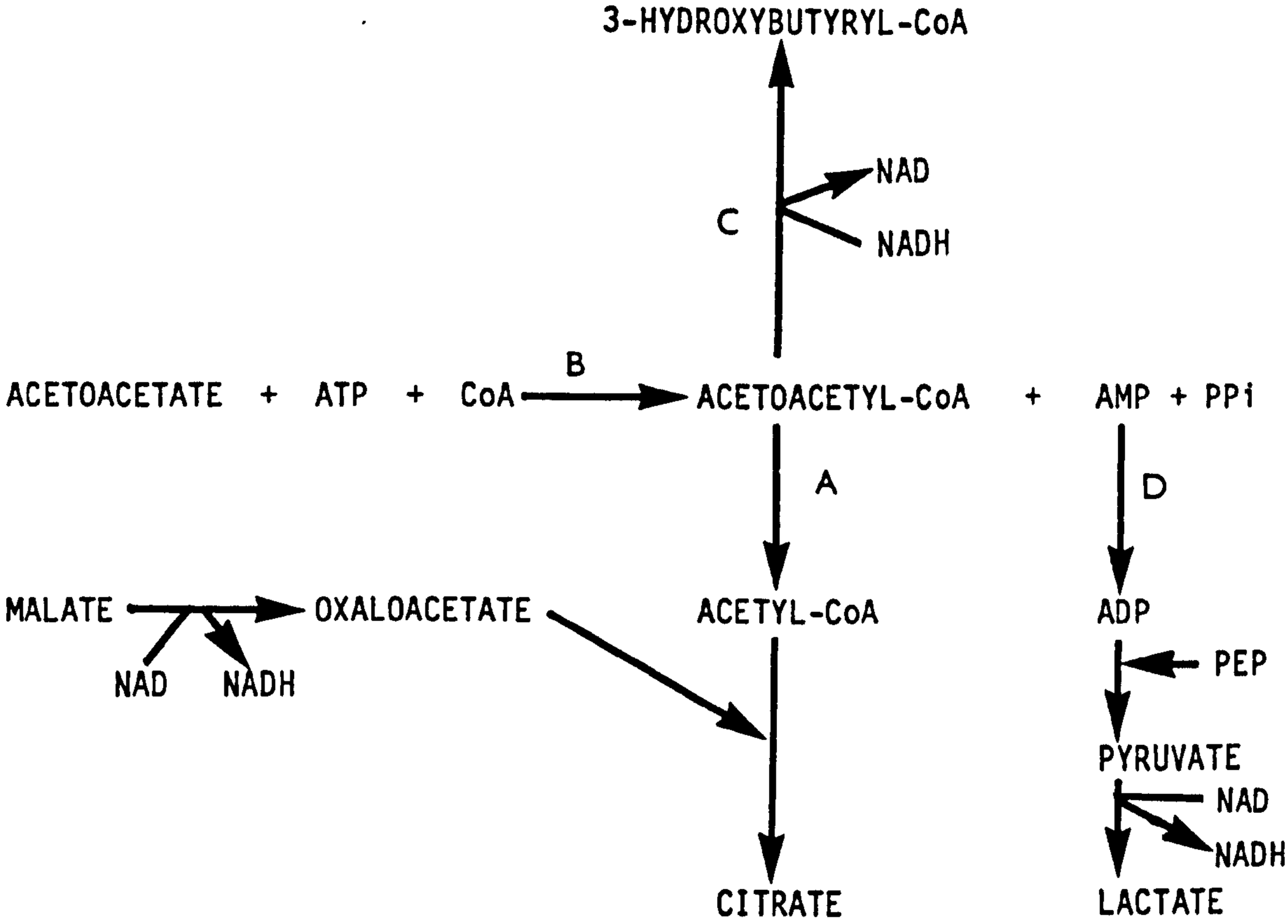


Figure 4.1 Methods Used for the Assay of Acetoacetyl-CoA Synthetase

(A). Acetyl-CoA Formation in the Presence of Beta-Ketothiolase. This linked assay indirectly monitors synthetase activity by utilising the beta-ketothiolase activity present in the crude extract, on the primary product of the reaction, acetoacetyl-CoA. The assay mixture (1 ml) contained Tris-HCl buffer (50 mM, pH 8.6), acetoacetate (0.2 mM), ATP (0.2 mM), CoASH (0.1mM), KCl (20 mM) and $MgCl_2$ (5 mM). The reaction was initiated by the addition of enzyme and incubated at 25°C for 5 min. The enzyme reaction was terminated by the addition of HCl (4 M, 20 µl). Following removal of the protein precipitate by centrifugation (2,500 x g, 15 min), the pH of the supernatant was adjusted to pH 8.0 - 8.5 with 1 M Tris (free base). The acetyl-CoA formed was assayed enzymatically with citrate synthase (1 unit) and malate dehydrogenase (1 unit) in the presence of malate (10 mM) and NAD (2 mM). The amount of NADH formed in the reaction (measured at 340 nm) is proportional to the acetyl-CoA formed initially.

(B). Enolate Formation. Once interference from beta-ketothiolase had been removed, synthetase activity could be directly assayed by monitoring the production of acetoacetyl-CoA (enolate form). The assay mixture was identical to that described in (A). Synthesis of the enolate form of acetoacetyl-CoA was monitored at 303 nm using an absorption coefficient for acetoacetyl-CoA of 21.4 x 10 1. mol^{-1} cm^{-1} (determined as described in section 5.3.5). The unit described in the text is defined as the amount of enzyme that, under the conditions of the assay, catalyses the formation of 1 µmole of acetoacetyl-CoA (enolate form) in 1 min at 25°C.

(C). NAD Formation. The assay mixture (1 ml) was identical to that described in (A), with the addition of NADH (0.1 mM) and L(+)-3-hydroxyacyl-CoA dehydrogenase (1 unit) from pig heart. The reaction was monitored at 25°C by following the rate of NADH oxidation (measured at 340 nm) during the conversion of acetoacetyl-CoA to 3-hydroxybutyryl-CoA.

(D). AMP Formation. AMP formation was monitored by the method described by Fukui et al. (1982). Additions to the reaction mixture described in (A) were as follows: phosphoenolpyruvate (1.5 mM), NADH (0.1 mM), pyruvate kinase from rabbit muscle (2 units), adenylate kinase from rabbit muscle (7 units) and lactate dehydrogenase from beef heart (7 units). The rate of AMP formation was indirectly monitored at 340 nm and 25°C due to the oxidation of NADH.

4.3 RESULTS

4.3.1 PURIFICATION OF ACETOACETYL-COA SYNTHETASE FROM M. TRICHOSPORIUM OB3B

The results of the purification scheme are presented in Table 4.1. The first step of this procedure resolved completely the synthetase activity from interference by beta-ketothiolase, allowing the subsequent assay of the enzyme by direct measurement of acetoacetyl-CoA formation. At pH 6.0 beta-ketothiolase is close to its iso-electric point as judged by its slow desorption from the DEAE-Sepharose column, following extensive washing with the equilibration buffer. The wash protocol was essential in resolving the two enzymes since contamination of the eluted synthetase with trace quantities of beta-ketothiolase was frequently observed when the elution of the column was initiated prior to incomplete desorption of beta-ketothiolase. The nature of the problem is highlighted if one compares the specific activities of both enzymes from the crude extract, where the activity of beta-ketothiolase (Chapter 5) is almost 300-fold higher than the corresponding synthetase activity from this organism. Subsequently, as a precautionary measure, desorption of beta-ketothiolase was routinely monitored during the wash stage and gradient elution of acetoacetyl-CoA synthetase activity proceeded only on complete desorption of this enzyme. The ensuing elution profile of acetoacetyl-CoA synthetase from the column of DEAE-Sepharose is illustrated in Figure 4.2.

STEP		VOLUME (ml)	PROTEIN (mg/ml)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)**	SPECIFIC ACTIVITY (µmol/min/mg)	PURIFICATION FACTOR (fold)	YIELD (%)
Cell free extract	*	314	18.5	5810	10.64	0.0018	1	100
DEAE-Sepharose	+	160	5.04	810	9.60	0.0119	6.5	90.2
Red H-3B Sepharose	+	20	1.28	26	6.95	0.2716	148.4	65.3
Sephacryl S-300	+	34	0.11	3.6	4.14	1.1370	621.3	38.9

* = Activity assay A: + = Activity assay B:

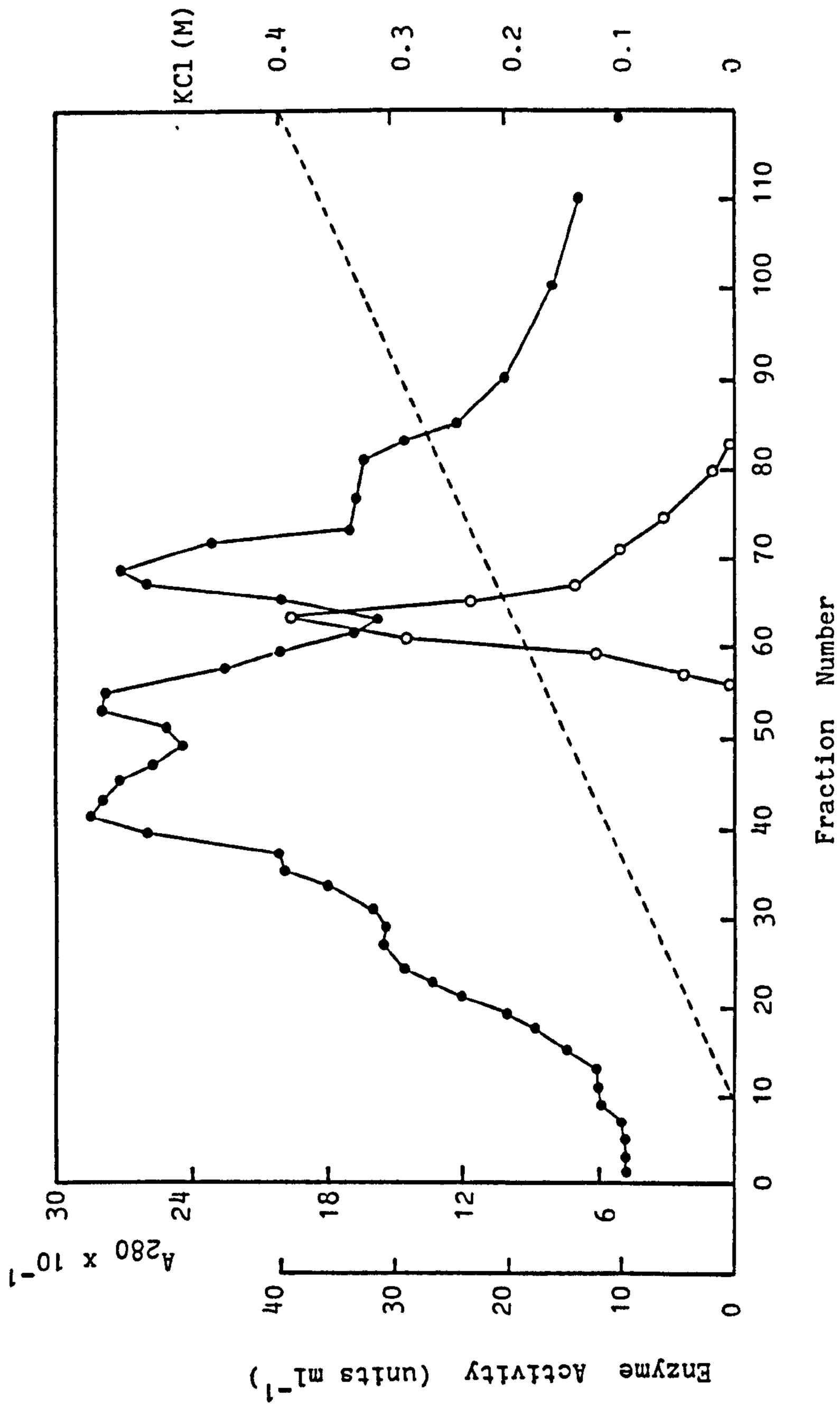
** 1 unit of enzyme = 1 µmol acetoacetyl-CoA formed $min.^{-1}$ at $25^{o}C$

Table 4.1 Purification of Acetoacetyl-CoA Synthetase from M. trichosporium OB3b

The table shows a summary of a typical purification from 200g of frozen cell paste. Experimental details are described in section 4.2.1.

Figure 4.2 Elution Profile of Acetoacetyl-CoA Synthetase from DEAE-Sepharose

The crude extract (5.8 g of protein) was applied to a column of DEAE-Sepharose (5 x 6.5 cm) equilibrated with potassium phosphate buffer (20 mM, pH 6.0). Elution was performed with a linear salt gradient (0 - 0.4 M KCl, 800 ml) at a flow rate of 60 ml.h^{-1}. Fractions of 8 ml were collected.(O) Acetoacetyl-CoA synthetase activity; (●) A_{280} ; (--) KCl (M).


KCl (M)
0.4
0.3
0.2
0.1
0
110
100
90
80
70
60
50
40
30
20
10
0
Fraction Number
30
24
18
12
6
0
$A_{280} \times 10^{-1}$
0
0
0
0
Enzyme Activity (units ml^{-1})
4
3
2
1
0

The triazine dye Sepharose conjugates, Procion Red H-3B and Cibacron Blue F3G-A, were each shown to bind acetoacetyl-CoA synthetase from the crude extract of M. trichosporium OB3b. The affinity of both dye-conjugate for the enzyme, however, was low (approximately 0.02 and 0.035 units ml^{-1} of Blue F3G-A and Red H-3B Sepharose respectively) and therefore, the suitability of triazine dye affinity chromatography as a first step in the purification scheme was inappropriate since a large volume of dye-conjugate would be required in order to bind adequate acetoacetyl-CoA synthetase for use in subsequent analysis. A requirement to reduce the number of proteins capable of binding to each dye-conjugate and subsequently increase the binding capacity of the respective column for the enzyme was effectively provided by the ion-exchange step described previously.

The binding capacity of Blue F3G-A (0.16 units/ml dye Sepharose gel) and Red H-3B Sepharose for acetoacetyl-CoA synthetase (0.28 units/ml dye-Sepharose gel) was substantially higher following the initial purification step. Indeed, by careful selection of eluant triazine dye affinity chromatography provided an excellent second step in the resolution of synthetase activity from contaminating protein. Cofactor elution of the enzyme from Red H-3B Sepharose or Blue F-3GA could be effected by addition of either ATP or AMP to the equilibration buffer. However, in contrast, CoASH was successful in desorbing the enzyme from Red H-3B Sepharose but ineffective in removing the enzyme bound by Blue F3G-A Sepharose. This probably reflects a difference in the point of interaction of acetoacetyl-CoA synthetase with the two dye-conjugates and further emphasises the importance of optimising the dye/eluant for each enzyme in order to

derive the most effective chromatographic resolution from "contaminating" proteins.

Examination of the elution profile of acetoacetyl-CoA synthetase following gel filtration (Figure 4.3) suggested that the enzyme was highly pure. Although the specific activity of the methanotrophic enzyme was considerably lower (1.14 units (mg protein)$^{-1}$) than that from the only other identified bacterial source of this enzyme, Z. ramigera I-16-M (52.2 units (mg protein)$^{-1}$) (Fukui et al., 1982), both the yield and the extent of purification of the enzyme compared favourably. The methanotrophic enzyme was stable for at least seventy two hours at 4^{o}C and stabilising agents were not required at any point in the purification scheme.

4.3.2 MOLECULAR WEIGHT OF ACETOACETYL-COA SYNTHETASE

The molecular weight of acetoacetyl-CoA synthetase was determined by its elution profile on Sephacryl S-300, previously standardised with known molecular weight proteins (Section 2.7). A molecular weight of 64,000 was determined for the native protein. (Figure 4.4). Owing to the small amount of protein produced from the purification of this enzyme, SDS-PAGE analysis of subunit composition could not be assessed during this study. Therefore, further analysis of this enzyme by gel elecrophoresis is required in order to estimate the subunit composition of the enzyme and, more importantly, to determine whether it exists as a single discrete protein at this level of purification.

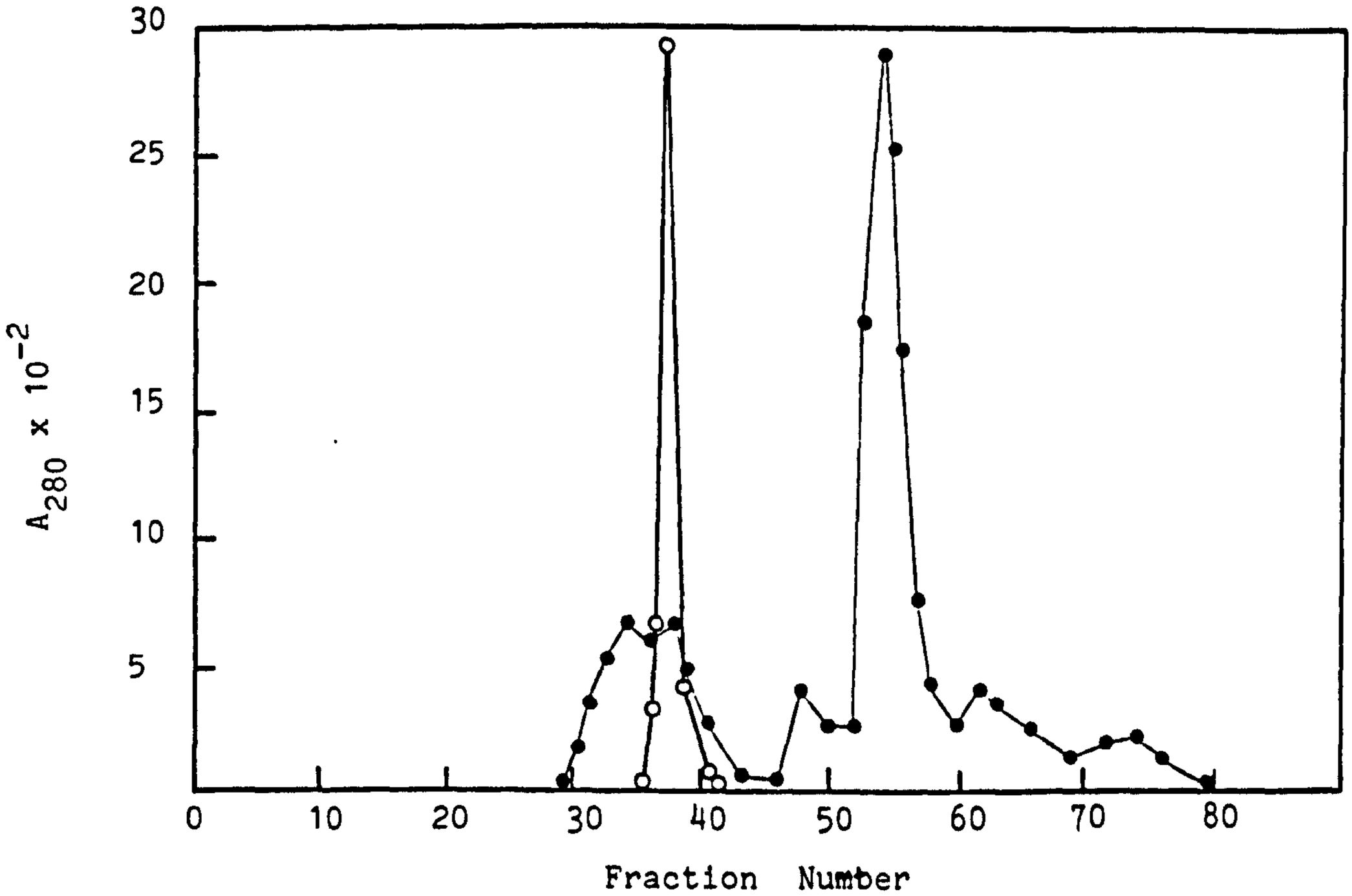


Figure 4.3 Elution Profile of Acetoacetyl-CoA Synthetase from Sephacryl S-300

A concentrated enzyme solution (3.6 mg of protein) was applied to a column of Sephacryl S-300 (2.5 x 60 cm) equilibrated with Tris-HCl buffer (50 mM, pH 7.5) at a flow rate of 30 ml.h^{-1}. Fractions of 5 ml were collected. (○) Acetoacetyl-CoA synthetase activity; (●) A_{280}.

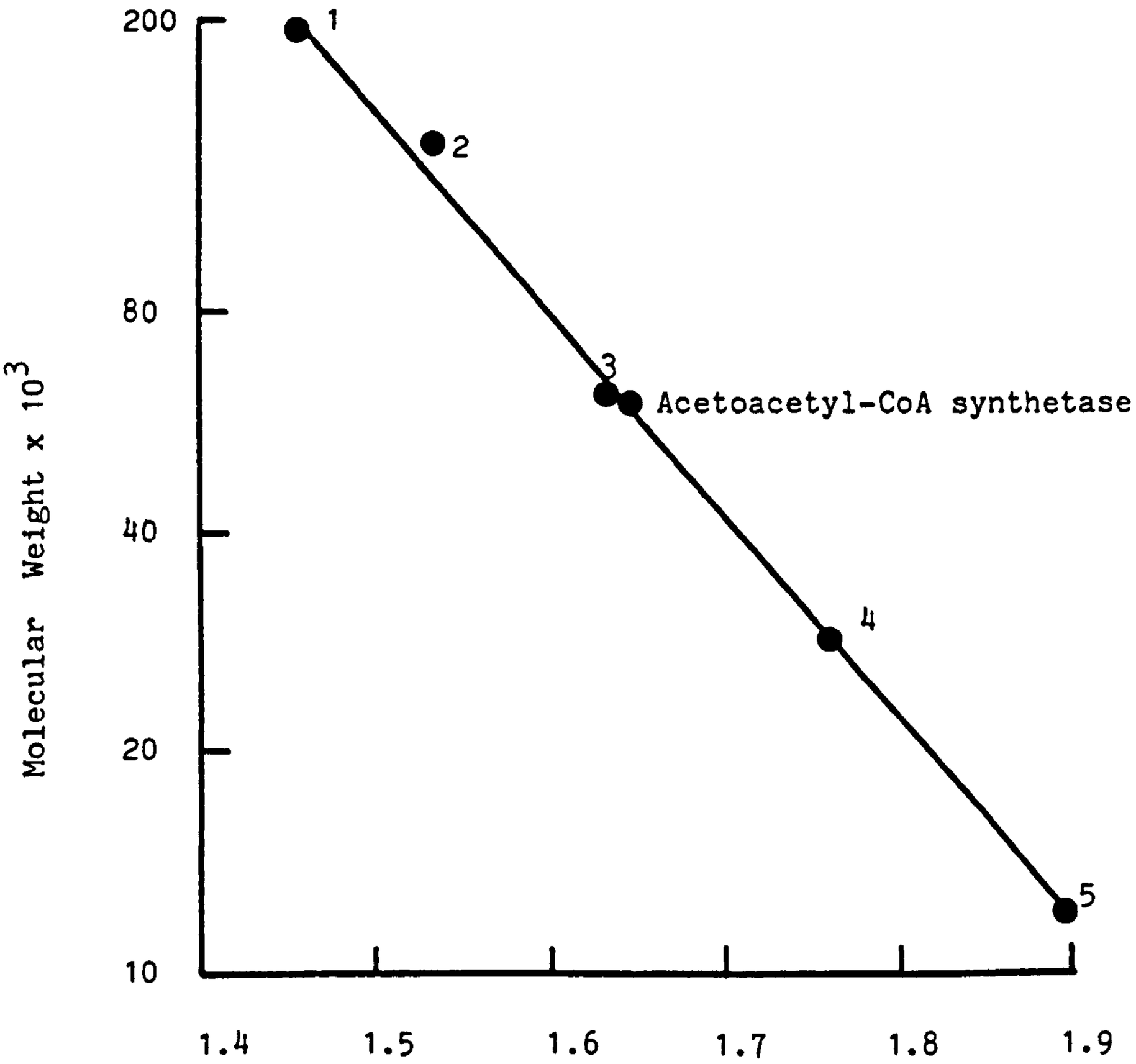


Figure 4.4 Determination of the Molecular Weight of Acetoacetyl-CoA Synthetase from M. trichosporium OB3b by Sephacryl S-300 Gel Filtration

The elution volume of acetoacetyl-CoA synthetase from a column of Sephacryl S-300 was compared to proteins of known molecular weight. 1. Beta-Amylase; 2. Alcohol Dehydrogenase; 3. Albumin, Bovine; 4. Carbonic Anhydrase; 5. Cytochrome C (see section 2.9 for further details).

4.3.3 OPTIMISATION OF ASSAY CONDITIONS FOR ACETOACETYL-COA SYNTHETASE

The pH optimum for purified acetoacetyl-CoA synthetase activity was 8.6 when measured by both methods (A) and (B) (Figure 4.5) in Tris-HCl buffer. Purified beta-ketothiolase, (Chapter 5) was utilised as part of the linked assay (Figure 4.1) in all measurements conducted by method (B).

The requirement of the enzyme for both a monovalent (Figure 4.6) and a divalent cation (Table 4.2) was absolute. Of the monovalent cations tested, potassium ions were optimum for the system. However, potassium could be replaced by either NH_4^+ and Cs^+ ions. Li^+ or Na^+ ions did not activate the enzyme. The specificity of the divalent cations ranked in the order $Mg^{2+} = Mn^{2+} > Ni^{2+} > Zn^{2+} > Ca^{2+} > Co^{2+}$ and Fe^{2+} ions were not able to stimulate synthetase activity.

4.3.4 SUBSTRATE SPECIFICITY OF ACETOACETYL-COA SYNTHETASE

In view of the low specific activity of acetoacetyl-CoA synthetase from M. trichosporium OB3b, the possibility that the enzyme might activate other organic acids in preference to acetoacetate was investigated. This was tested with a range of organic acids, using the AMP formation assay method (method D). Of the organic acids tested, the enzyme activated only acetoacetate. Substrates tested (0.01 mM - 2mM), which were not activated, included acetate, n-butyrate, crotonate, propionate, succinate, malate, glyoxylate, glycollate, glutarate, mesoconate and formate.

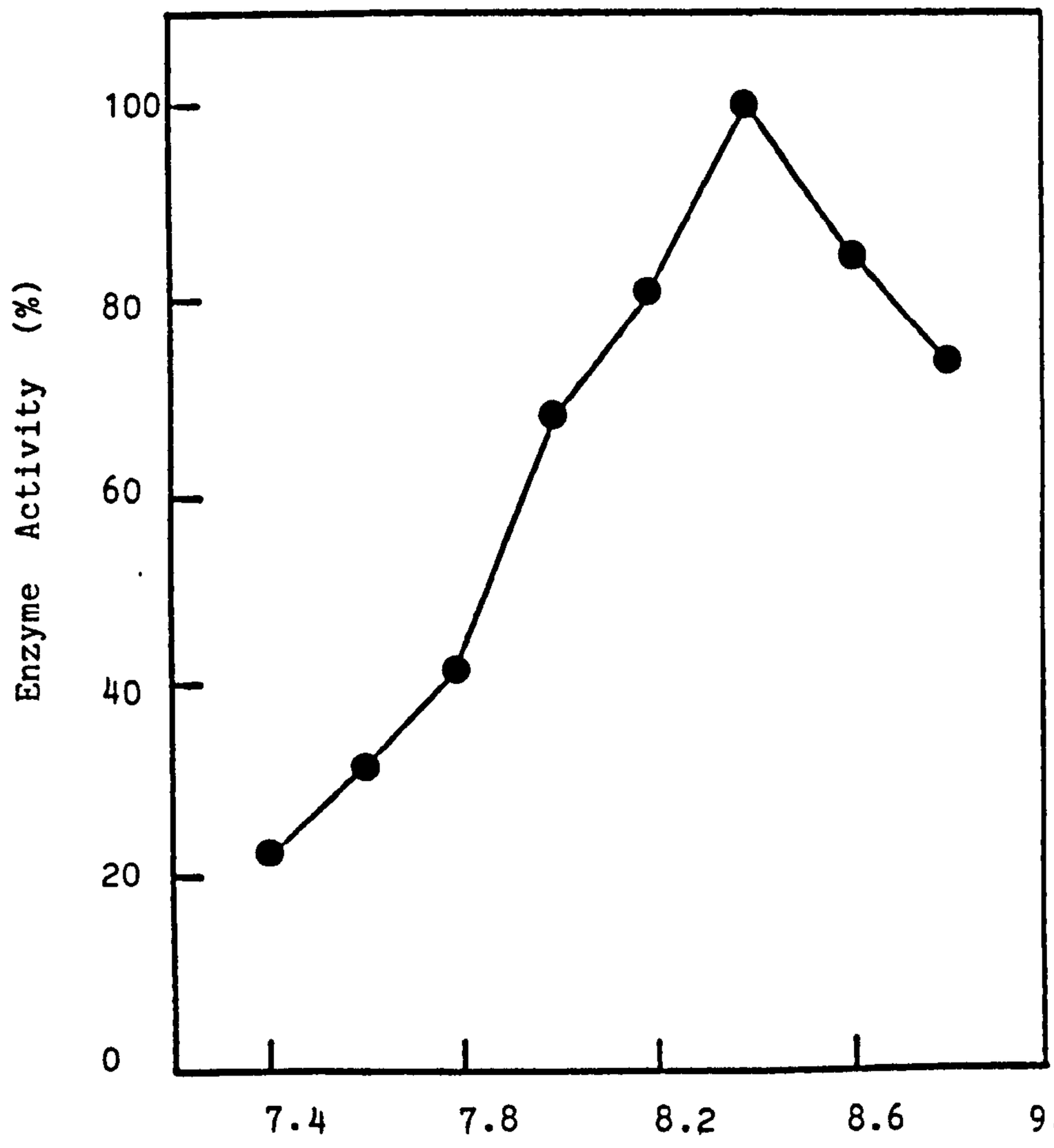


Figure 4.5 Dependence of the Activity of Acetoacetyl-CoA Synthetase on pH

Enzyme activity was measured by monitoring the production of the magnesium-enolate form of acetoacetyl-CoA (method B), using purified acetoacetyl-CoA synthetase (5.5 µg protein). (●) Acetoacetyl-CoA synthetase activity.

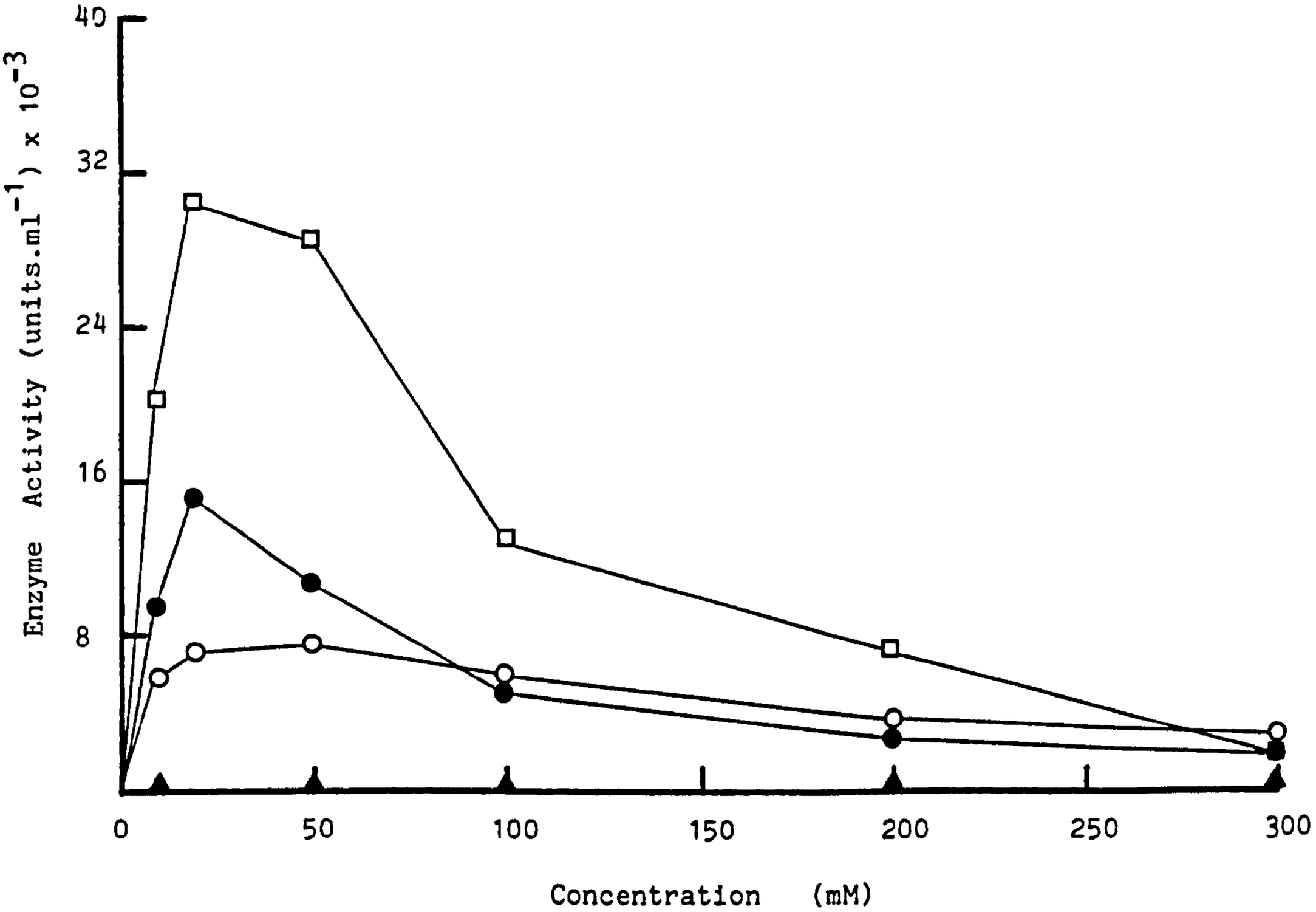


Figure 4.6 Effect of the Concentration of Monovalent Cations on Acetoacetyl-CoA Synthetase Activity from M. trichosporium OB3b

The enzyme activity was measured by monitoring enolate formation of acetoacetyl-CoA (method B) using purified acetoacetyl-CoA synthetase (5.5 µg protein) in the presence of increasing concentrations of monovalent cations (chloride salt). (□) K^+; (●) NH_4^+; (○) Cs^+; (▲) Li^+ or Na^+.

Divalent Cation (1 mM)	Activity (%)
Mg^{2+}	100
Mn^{2+}	100
Ni^{2+}	87
Zn^{2+}	87
Ca^{2+}	70
Co^{2+}	0
Fe^{2+}	0

Table 4.2 Divalent Cation Requirement for Acetoacetyl-CoA Synthetase Activity from M. trichosporium OB3b

The enzyme activity was assayed by measuring NAD formation (method C) by using the purified acetoacetyl-CoA synthetase (5.5 μg protein) and 3-hydroxyacyl-CoA dehydrogenase from pig heart (1 unit). The concentration of divalent cation (chloride salt) was 5 mM; the monovalent cation (potassium), remained constant (50 mM) in each assay conducted.

4.3.5 ALTERNATIVE PATHWAYS OF ACETOACETATE REACTIVATION

Initial studies on the mechanism of acetoacetate reactivation in M. trichosporium OB3b were aimed at demonstrating activity of acetoacetate:succinate CoA-transferase. However, despite an exhaustive study, all attempts at detecting activity of this enzyme were unsuccessful. The various strategies employed in this respect included:-

(1) The preparation of crude extracts from cells grown under conditions which result in mobilisation of PHB deposits (see chapter 7)

(2) Crude extracts were prepared by both sonication and by high pressure disruption, with and without the addition of a sulphydryl reagent.

(3) Enzyme activity was assayed in the crude extract and cell wall/membrane fraction.

(4) Enzyme activity was assayed in the pH range 5.0 - 9.0 (Tris-HCl and phosphate buffers), in both directions, at various concentrations of each component in the assay system.

The possibility that succinate may not be the compound activated by the transfer of CoASH from acetoacetyl-CoA was investigated. The following compounds were inactive as replacements for succinate in extracts prepared as indicated in (1): malate, propionate, crotonate, acetate, glycollate, glyoxylate, D(-)-3-hydroxybutyrate, formate and butyrate.

4.3.6 INHIBITION STUDIES ON ACETOACETYL-COA SYNTHETASE

Acetoacetyl-CoA synthetase was not inhibited by either substrates (acetoacetate, CoA or ATP) or products (AMP or acetoacetyl-CoA) in the direction of acetoacetate activation. Similarly activity of the enzyme was not diminished in the presence of acetone (10 mM), acetyl-CoA (5 mM), D(-)-3-hydroxybutyrate (10 mM), nicotinamide nucleotides (NADPH, NADH, NADP and NAD; all at 1 mM) or intermediates of the TCA cycle (10 mM). All tests were carried out under conditions where each component involved in the activation reaction was non-saturating.

4.4 DISCUSSION

This study provides the first description of acetoacetyl-CoA synthetase in a methanotrophic (or methylotrophic) species and only the second report of a bacterial source of this enzyme. It therefore represents a divergence from the classical pathway of PHB mobilisation (Senior & Dawes, 1973), in that no other enzyme activity involved in the activation of acetoacetate (including a number of CoA transferases) could be demonstrated in this organism. This study, however, did not resolve the question as to whether the enzyme activity responsible for the reactivation of acetocetate was attributable to a single protein. Although, the final gel filtration step in the purification sequence resolved the enzyme to a single peak, which corresponded exclusively to acetoacetyl-CoA synthetase activity, this did not provide an absolute criteria of enzyme purity. Consequently, further unambiguous evidence is required before this situation can be resolved.

The acetoacetyl-CoA synthetase purified from M. trichosporium OB3b resembles the analogous enzyme from Z. ramigera I-16-M, in many respects. Both enzymes have an absolute requirement for ATP and CoASH in the presence of a monovalent and divalent cations for optimum activity. The only minor differences occur in the extent of enzyme activation in the presence of each cation tested. Although the substrate specificity of both enzymes is marked towards acetoacetate, the synthetase from Z. ramigera also activates L(+)-3-hydroxybutyrate (15 % as active as acetoacetate under the same assay conditions). Both

enzymes are AMP-forming acetoacetate:CoA ligases.

The specific activity of the methanotrophic synthetase appears to be low, both in comparison with the other enzymes on the pathway of PHB mobilisation and the activity of the enzyme from Z. ramigera I-16-M. The relative activities are indicated below:

Organism	3-HBD	Acetoacetyl-CoA synthetase	Beta-ketothiolase
M. trichosporium OB3b	326	1.14	186
Z. ramigera I-16-M	123	52.2	136

Enzyme activities are expressed in units (mg. protein)$^{-1}$.

These results, at least, indicate that the activation of acetoacetate in M. trichosporium OB3b may be the rate limiting step in the mobilisation of PHB. Furthermore, the question is posed as to whether the activity of this enzyme is sufficient to account for the mobilisation and use of PHB as both a carbon and energy reserve, for instance in the sporulation process. Certainly the ability of storage polymers to act as carbon sources for protein synthesis in obligate methanotrophs has been intimated by Linton and Cripps (1978), who demonstrated the uptake of ^{35}S during the metabolism of a polyglucose storage polymer by a Type 1 methanotroph.

Three possible explanations are advanced to account for the low activity of acetoacetyl-CoA synthetase in this methanotroph.

(i). Acetoacetate may be activated through a metabolic route, other than the two highlighted in this chapter. Under these circumstances, low activity synthetase might serve to replenish a metabolic pool(s) distinct from the mainstream metabolic pathway. With this in mind, it is interesting to note that both acetoacetyl-CoA synthetase (cytosolic) and acetoacetate:succinate CoA-transferase (mitochondrial) activity have been detected in the same mammalian tissue. Although both enzymes effect the conversion of acetoacetate to its CoA ester they are, nevertheless, thought to perform distinctly dissimilar metabolic roles (Robinson & Williamson, 1980). Acetoacetyl-CoA transferase activity is generally more than ten-fold higher than the corresponding synthetase activity, and is thought to be associated with the pathway for oxidation of ketone bodies (via the TCA cycle). In contrast, acetoacetyl-CoA synthetase activity is thought to be primarily associated with lipid biosynthesis.

(ii). The enzyme activity <u>in vivo</u> might be substantially higher either through induction of enzyme activity or by interaction with specific activators.

(iii). At present, the most likely explanation appears to centre on the possible physiological role of PHB in this organism. If PHB was mobilised almost exclusively for the generation of reduced pyridine nucleotide, by the action of 3-HBD, then the necessity to reactivate the resulting acetoacetate would be diminished. This would rationalise

the presence of an acetoacetate decarboxylase activity in this organism (Thompson et al., 1976; Best, 1982), and fits the proposals presented on the role of 3-HBD in PHB mobilisation (Chapter 3). This postulate envisages a major role for PHB in providing reducing equivalents, during a demand on the NADH pool, and a minor role in providing carbon equivalents through further metabolism of acetyl-CoA. These proposals are further highlighted in Chapter 7, where metabolite flux through the pathway is investigated.

CHAPTER FIVE

THE PURIFICATION AND PROPERTIES OF BETA-KETOTHIOLASE FROM M. TRICHOSPORIUM OB3B

5.1 INTRODUCTION

Two distinct forms of beta-ketothiolase have been isolated and described to date. Although both enzymes use acetoacetyl-CoA as a substrate, they differ in the chain length of the beta-ketoacyl-CoA esters which they can cleave. Short chain specific beta-ketothiolases (EC 2.3.1.9) are highly specific for the four carbon (C_4) substrate acetoacetyl-CoA, and catalyse the following reaction (equation 5.1)

$$CH_3CO.SCoA + CH_3CO.SCoA \rightleftharpoons CH_3CO.CH_2CO.SCoA + CoASH \quad ...(5.1)$$

Long chain specific beta-ketothiolases (acyl-CoA : acetyl-CoA C-acetyl transferase; EC 2.3.1.16) have a wide substrate specificity and are active on CoA thioesters of 4-16 carbon atoms. The cleavage of beta-ketoacyl-CoA esters (n carbons) by CoASH to produce acetyl-CoA and a saturated acyl-CoA ester (n-2 carbon atoms) is illustrated below (equation 5.2).

$$R\text{-}CH_2CO.CH_2CO.SCoA + CoASH \rightleftharpoons R\text{-}CH_2CO.SCoA + CH_3CO.SCoA \quad ..(5.2)$$

where R = 4 - 16 carbon atoms.

The reaction was first postulated by Lynen et al. (1951), and shortly thereafter direct experimental evidence was obtained independently in three laboratories (Lynen et al., 1952; Green et al., 1953; Stern et al., 1953).

Beta-ketothiolase is ubiquitous in nature and has been studied in a wide variety of cells. These include both PHB producing bacteria (Oeding & Schlegel, 1973; Senior & Dawes, 1973; Nishimura et al., 1978), non-PHB producing bacteria (Mazzai et al., 1970; Berndt & Schlegel, 1975), yeast (Kornblatt & Rudney, 1971a and 1971b), plants (Cooper & Beevers, 1969) and most mammalian tissues, particularly in metabolically active organs such as the liver, heart and kidney (Gehring & Riepertinger, 1968; Clinkenbeard et al., 1973; Huth et al., 1974; Middleton, 1973).

Beta-ketothiolases are involved in many important metabolic pathways including the breakdown of fatty acids, the biosynthesis of sterols and the formation of ketone bodies, and play an important role in the regulation of lipid metabolism. Their physiological functions are highlighted below.

5.1.1 LONG CHAIN SPECIFIC BETA-KETOTHIOLASES

Long chain specific beta-ketothiolase is thought to be closely associated with the beta-oxidation cycle, where it forms the last in a series of reactions, involving acyl-CoA dehydrogenase, enoyl-CoA hydratase and beta-hydroxyacyl-CoA dehydrogenase; the sequential action of each enzyme produces beta-ketoacyl-CoA ester, which is subsequently cleaved by long chain specific beta-ketothiolase to yield acetyl-CoA and a saturated acyl-CoA thioester shortened by two carbons. The shortened acyl-CoA thioester can undergo another cycle of oxidation starting with the reaction catalysed by acyl-CoA dehydrogenase.

Although long chain specific beta-ketothiolases have been isolated and studied extensively from mammalian tissue (Seubert et al., 1968; Middleton, 1972), relatively few studies have been conducted on the enzyme from bacteria. Work by Overath et al. (1969) with E. coli has revealed that the enzymes of beta-oxidation were co-induced when the cells were grown on media containing oleate as the sole carbon source. Genetic and biochemical analysis of mutants which were unable to grow on oleate, indicated that the genes for the enzymes of beta-oxidation and long chain beta-ketothiolase were closely linked and probably on the same operon.

5.1.2 SHORT CHAIN SPECIFIC BETA-KETOTHIOLASES

In contrast to the long chain specific beta-ketothiolases the short chain specific enzymes are known to participate in a number of metabolic pathways; these are highlighted below.

5.1.2.1 Role in Sterol Formation

Two electrophoretically distinct short chain specific beta-ketothiolases have been identified and partially characterised from S. cerevisiae (Kornblatt & Rudney, 1971a; 1971b). One form of the enzyme was located in the mitochondrion, whilst the other form was predominantly cytosolic. Kinetic studies on the cytosolic enzyme revealed that it was markedly different from the mitochondrial equivalent. The enzyme was inhibited by both its substrates (CoASH and acetoacetyl-CoA) which suggested that its mode of operation was in the direction of acetoacetyl-CoA synthesis and subsequently sterol

biosynthesis via mevalonic acid. Absence of substrate inhibition coupled with an exceptionally low K_m for CoASH suggested that the mitochondrial enzyme operated primarily in the direction of acetyl-CoA formation and subsequently energy metabolism via the TCA cycle.

Kinetic analysis on short chain beta-ketothiolases from mammalian cells have ascribed similar physiological roles for isoenzymes of this enzyme (Middleton, 1972 and 1974; Huth et al., 1975).

5.1.2.2 Role in Butyrate Metabolism

Beta-ketothiolase from C. pasteurianum was thought to be predominantly active in the direction of acetoacetyl-CoA formation (Berndt & Schlegel, 1972). These conclusions were based on a detailed kinetic analysis of this enzyme which showed that it was susceptable to a double substrate (CoASH and acetoacetyl-CoA) inhibition in the direction of acetoacetyl-CoA cleavage. In this organism beta-ketothiolase appears to act as a control point on the pathway to remove excess reducing equivalents via butyrate formation, during fermentative energy generation.

5.1.2.3 Role in PHB Metabolism

The physiological role of beta-ketothiolase from bacteria capable of metabolising PHB is distinctly dissimilar from that described for the same enzyme activity in yeast and clostridia. Beta-ketothiolase from PHB producing bacteria participates in both the formation and cleavage of acetoacetyl-CoA during the synthesis and mobilisation of PHB respectively. This property is subsequently reflected in the kinetics and overall regulation of the enzyme. Kinetic studies on the condensation reaction suggest that it is regulated by the relative levels of CoASH. This is true of all PHB producing bacteria studied to date (Oeding & Schlegel, 1973; Senior & Dawes, 1973; Nishimura et al., 1978); the reaction is not inhibited by physiological concentrations of other metabolites.

In the direction of PHB mobilisation, the cleavage of acetoacetyl-CoA by beta-ketothiolase serves to complete the cyclical pathway of PHB metabolism. The enzyme from A. beijerinkii (Senior & Dawes, 1973) and A. eutrophus H16 (Oeding & Schlegel, 1973) were each susceptable to substrate (acetoacetyl-CoA) inhibition. However, this was dependent upon the ratio of acetoacetyl-CoA/CoASH since inhibition was effectively reduced by increasing the concentration of CoASH. In contrast, the beta-ketothiolase from Z. ramigera I-16-M was inhibited by both NADPH and NADH only (Nishimura et al., 1978).

5.1.3 MECHANISM OF ACTION OF BETA-KETOTHIOLASE

The observation that beta-ketothiolase is inactivated by various sulphydryl blocking reagents led Lynen (1953) to propose a reaction mechanism for the action of beta-ketothiolase. This was based on the assumption that an active site sulphydryl residue participated directly in the catalytic reaction of the enzyme and involved the formation of an acyl-enzyme complex. A similar mechanism was proposed by Goldman (1954) on purely kinetic data (equations (5.3) and (5.4))

$$CH_2CO.CH_2CO.SCoA + {}^{-}S\text{-}Enz \rightleftharpoons CH_3CO.SCoA + CH_3CO.S\text{-}Enz \quad ...(5.3)$$

$$CH_2CO.S\text{-}Enz + CoAS^{-} \rightleftharpoons CH_3CO.SCoA + {}^{-}S\text{-}Enz \quad ...(5.4)$$

An acetyl-enzyme complex was subsequently shown to be formed between acetyl-CoA and a reactive sulphydryl residue at the active site of the enzyme (Gehring & Harris, 1968, 1970).

Studies conducted on the enzyme from S. cerevisiae (Kornblatt & Rudney, 1971a) have shown that it is susceptable to inactivation by $NaBH_4$ in the presence of either acetoacetyl-CoA or acetyl-CoA. This observation was consistent with the formation of a Schiff base intermediate (a ketamine) during the enzymatic reaction and led to the postulate that there was a catalytically important amino group at the active site of the enzyme. A reaction mechanism which proceeds via a ketamine intermediate and also accounts for the participation of a sulphydryl group was presented by Kornblatt and Rudney (1971a).

Although the mechanism of action of beta-ketothiolase has been

elucidated largely as a result of studies on mammalian sources of this enzyme, the kinetic data and inactivation studies carried out on bacterial beta-ketothiolases are consistent with the mechanism highlighted above (Oeding & Schlegel, 1973; Senior & Dawes, 1973; Nishimura et al., 1978).

This chapter describes the purification and characterisation of beta-ketothiolase from M. trichosporium OB3b. The study also reveals information on the regulation of PHB metabolism in this organism together with general information on the control of carbon flux via acetyl-CoA/acetoacetyl-CoA, an area of intermediary metabolism which has received scant attention in methanotrophs (or methylotrophs) to date.

5.2 EXPERIMENTAL

5.2.1 PROTOCOL FOR THE PURIFICATION OF BETA-KETOTHIOLASE FROM M. TRICHOSPORIUM OB3B.

Frozen cells (80 g) were thawed, resuspended in potassium phosphate buffer (20 mM, pH 6.5 containing dithiothreitol (1 mM), 150 ml) at room temperature. The cell free extract was prepared as described previously (section 2.5), with the exception that the final pH was adjusted to 6.5. All purification procedures were carried out at 4°C where possible. In addition, each buffer used in the purification sequence contained dithiothreitol (1 mM).

Step 1: Chromatography on DEAE-Sepharose

The cell free extract was applied to a column of DEAE-Sepharose (5 x 6.5 cm), preequilibrated with potassium phosphate buffer (20 mM, pH 6.5). The column was washed with equilibration buffer (250 ml) to remove unbound protein and the enzyme eluted with a linear gradient (400 ml) of potassium chloride (0-0.5 M) in the equilibration buffer. Elution of the enzyme proceeded at a flow rate of 60 ml. h^{-1}. Active fractions (5 ml) were pooled and dialysed by diafiltration (Amicon hollow fibre cartridge, 30,000 molecular weight cut off) against five volumes of potassium phosphate buffer (20 mM, pH 7.0,).

Step 2: Chromatography on Procion Red H-3B Sepharose

The dialysate from the first step was applied to a column of Procion Red H-3B (2.5 x 14 cm), preequilibrated with potassium phosphate buffer (20 mM, pH 6.5). The column was washed with equilibration buffer (100 ml) and the enzyme eluted, in an upwards direction using the same buffer containing CoASH (0.5 mM, 10 ml). Following application of CoASH, the column was washed with equilibration buffer (50 ml) until elution of the enzyme was complete. All enzyme activity was collected, pooled and dialysed by diafiltration against phosphate buffer (20 mM, pH 7.0, 10 vol) to remove all traces of CoASH. The enzyme preparation was used in subsequent analysis.

5.2.2 ENZYME ASSAYS

The optimum conditions for beta-ketothiolase activity, from M. trichosporium OB3b, was determined spectrophotometrically at 25° C in a total reaction volume of 1 ml (1 cm light path). These were found to be as follows:

A. Condensation reaction (final concentration): Tris-HCl buffer (100 mM, pH 7.5); acetyl CoA (5 mM); commercial 3-hydroxyacyl-CoA dehydrogenase (1 unit); NADH (0.15 mM); purified enzyme preparation (10.7 µg of protein). The reactants were incubated for 2 min before addition of acetyl-CoA. The reaction was monitored by measuring the decrease in absorbance at 340 nm due to the reoxidation of NADH by 3-hydroxyacyl-CoA dehydrogenase, following the formation of acetoacetyl-CoA. Using this assay, one unit of enzyme was defined as

the amount of enzyme that, under the conditions of the assay, catalyses the oxidation of 1 µmole of NADH in 1 min. at 25°C; this is equivalent to the formation of 1 µmole of acetoacetyl-CoA during the same period.

B. Acetoacetyl-CoA cleavage (final concentration): Tris-HCl buffer (100 mM, pH 8.35, containing 20 mM $MgCl_2$); CoASH (80 µM); acetoacetyl-CoA (60 µM); purified enzyme preparation (10.7 µg of protein). The reaction was monitored by measuring the decrease in absorbance at 303 nm due to disappearance of the Mg^{2+}-enol complex of acetoacetyl-CoA. The molar extinction coefficient of acetoacetyl-CoA was determined to be 18.8×10^3 $l.mol.^{-1}$ $cm.^{-1}$ under these conditions. Using this assay, one unit of enzyme was defined as the amount of enzyme that, under the conditions of the assay, catalyses the cleavage of 1 µmole of acetoacetyl-CoA, to form two molecules of acetyl-CoA, in one minute at 25°C.

5.2.3 DETERMINATION OF THE MOLAR EXTINCTION COEFFICIENT OF ACETOACETYL-COA AT DIFFERENT PH´S

The absorption (E_{303}) of a sample of acetoacetyl CoA (30 µM) in Tris-HCl buffer (0.1 M, containing 20 mM $MgCl_2$) was measured at different pH´s. The molar extinction coefficient was subsequently calculated from the Beer-Lambert relationship as follows:

Example

10 µl of acetoacetyl-CoA (3×10^{-3} M) in 1 ml buffer gave an absorbance of 0.147 at pH 7.3,

$$[\text{Acetoacetyl-CoA}] = \frac{\text{Absorption}}{E_{303}}$$

$$3 \times 10^{-3} = \frac{0.147 \times 100}{E_{303}}$$

$$E_{303} = \frac{0.147 \times 100}{3 \times 10^{-3}}$$

$$= 4.9 \times 10^{3}\ \text{l.mol}^{-1}\ \text{cm}^{-1}$$

5.3 RESULTS

5.3.1 PURIFICATION OF BETA-KETOTHIOLASE FROM M. TRICHOSPORIUM OB3B

The scheme developed for the purification of beta-ketothiolase from M. trichosporium OB3b resulted in the resolution of this enzyme to near homogeneity in two steps, as indicated by SDS polyacrylamide gel electrophoresis (Figure 5.2). A summary of the chromatographic protocol, which produced a 276-fold purification of the enzyme with a 68 % yield, is presented in Table 5.1.

The use of triazine dye affinity chromatography in the purification of CoA-dependent enzymes has been largely unexploited to date and limited to but a few examples (White & Jencks, 1970 and 1976; Tormanen et al., 1976). Certainly, no information on the interaction of any triazine dye with CoA-dependent beta-ketothiolase has been published to date. This was considered somewhat surprising in view of the broad specificity of these dyes for enzymes requiring adenine containing cofactors (Haff & Easterby, 1978) and the enormous potential of affinity chromatography for the purification of specific enzymes. Therefore, as part of a preliminary investigation into the most effective protocol for the purification of beta-ketothiolase from M. trichosporium OB3b, a number of triazine dye-Sepharose conjugates were screened to determine the ability of each in binding this enzyme. The range of triazine dyes examined are shown in Table 5.2.

STEP	VOLUME (ml)	PROTEIN (mg/ml)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)*	SPECIFIC ACTIVITY (µmol/ min/mg)	PURIFICATION FACTOR (fold)	YIELD (%)
Cell free extract	180	8.26	1488	980	0.66	1	100
DEAE-Sepharose	82	0.60	48.8	803	16.32	24.8	82
Red H-3B Sepharose	75	0.04	2.9	546	182	276	68

* 1 unit = 1 µmole acetoacetyl-CoA cleaved $min.^{-1}$ at $25^{o}C$

Table 5.1 Purification of Beta-ketothiolase from M. trichosporium OB3b

The table shows a summary of a typical purification from 80g of frozen cell paste.

Experimental details are described in section 5.2.1.

Figure 5.2 SDS Polyacrylamide Gel Electrophoresis of Beta-Ketothiolase from M. trichosporium OB3b

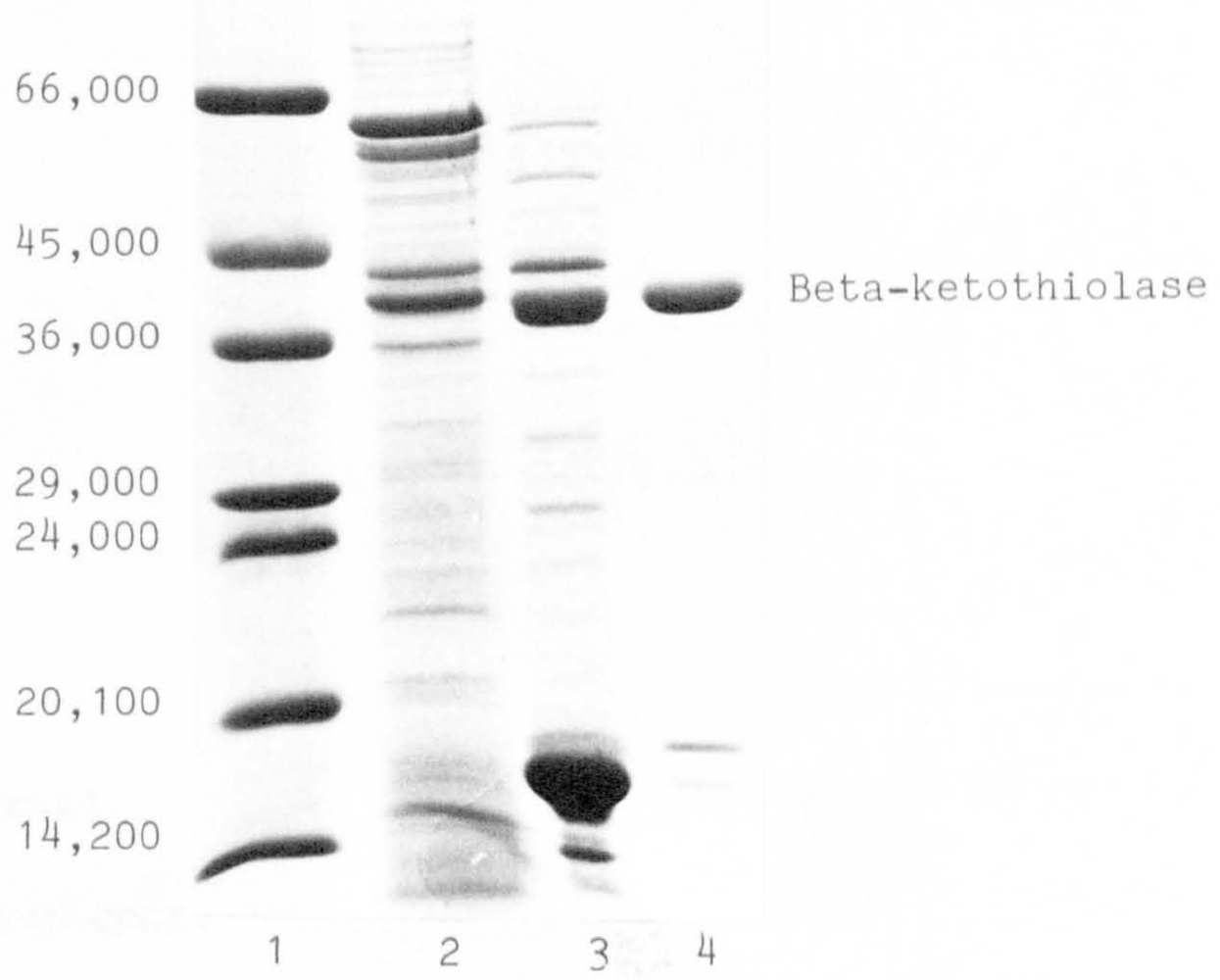
Samples from each step in the purification sequence were electrophoresed on 12 % gels by the method of Laemmli (1970) (section 2.10). Proteins were stained with Coomassie brilliant blue.

Lane 1, molecular weight markers (25 µg);

Lane 2, Crude extract (20 µg);

Lane 3, DEAE-Sepharose eluate (20 µg);

Lane 4, Red H-3B eluate (10 µg).


66,000
45,000
36,000
29,000
24,000
20,100
14,200
Beta-ketothiolase
1
2
3
4

Sepharose CL-6B Conjugate	Recovered Activity (%)	
	Buffer Wash	Buffer + 0.5 M KCl
Procion Orange H-2R	92	N.D.
Orange MX-2R	0	85
Yellow MX-4G	85	N.D.
Yellow M4RF	95	N.D.
Green H-4EBMX	0	72
Brown 4-RD	78	15
Blue MX-2G	93	N.D.
Blue MX-2R	5	81
Blue F3-GA	0	88
Red H-3B	0	81
Red MX-8B	0	90
Red H-8B	0	83
Sepharose CL-6B (control)	93	N.D.

Abbreviation: N.D., not done.

Table 5.2 Binding Studies of Beta-Ketothiolase from M. trichosporium OB3b on Sepharose CL-6B Dye Conjugates

Crude extract obtained from M. trichosporium OB3b (1 ml) containing 2 units of beta-ketothiolase was applied to each column (5 ml) of dye Sepharose conjugate, equilibrated in phosphate buffer (20 mM, pH 6.5). Columns were washed with the same buffer (20 ml) followed by buffer containing potassium chloride (0.5 M). Enzyme activity was determined as described in section 5.2.2.

Of the twelve dye-conjugates examined in this study, eight were successful in binding beta-ketothiolase from the crude extract of M. trichosporium OB3b. Although triazine dyes are known to differ in their selectivity and subsequent purification of specific enzymes, for the purpose of this study, two dye conjugates only were subjected to a detailed re-examination in order to ascertain the suitability of this technique in the purification of beta-ketothiolase. The two dyes chosen for further analysis were Procion Red H-3B and Cibacron Blue F3G-A, each of which were coupled to Sepharose CL-6B.

Following a study to determine the binding capacity of each dye-conjugate for beta-ketothiolase it became apparent that triazine dye affinity chromatography was unsuitable as a first step in the purification sequence. This was largely attributable to the low binding capacity of each dye-conjugate for this enzyme (0.5 and 0.4 units/ml Sepharose gel for Red H-3B and Blue F3G-A respectively). These results were not atypical for the two dye conjugates examined, since comparable results were obtained on a brief re-examination of the remaining six dye-conjugates shown to bind beta-ketothiolase in Table 5.2. Clearly the results of this study suggested that an initial purification of beta-ketothiolase was required to circumvent the use of inordinate volumes of dye-conjugate to bind the enzyme.

DEAE-Sepharose chromatography at pH 6.5 provided a satisfactory initial purification step in the preparation of beta-ketothiolase for the second step of affinity chromatography. At this pH beta-ketothiolase was close to its isoelectric point as judged by the

low ionic strength required to elute the enzyme from this column. The elution profile of beta-ketothiolase from DEAE-Sepharose is illustrated in Figure 5.3.

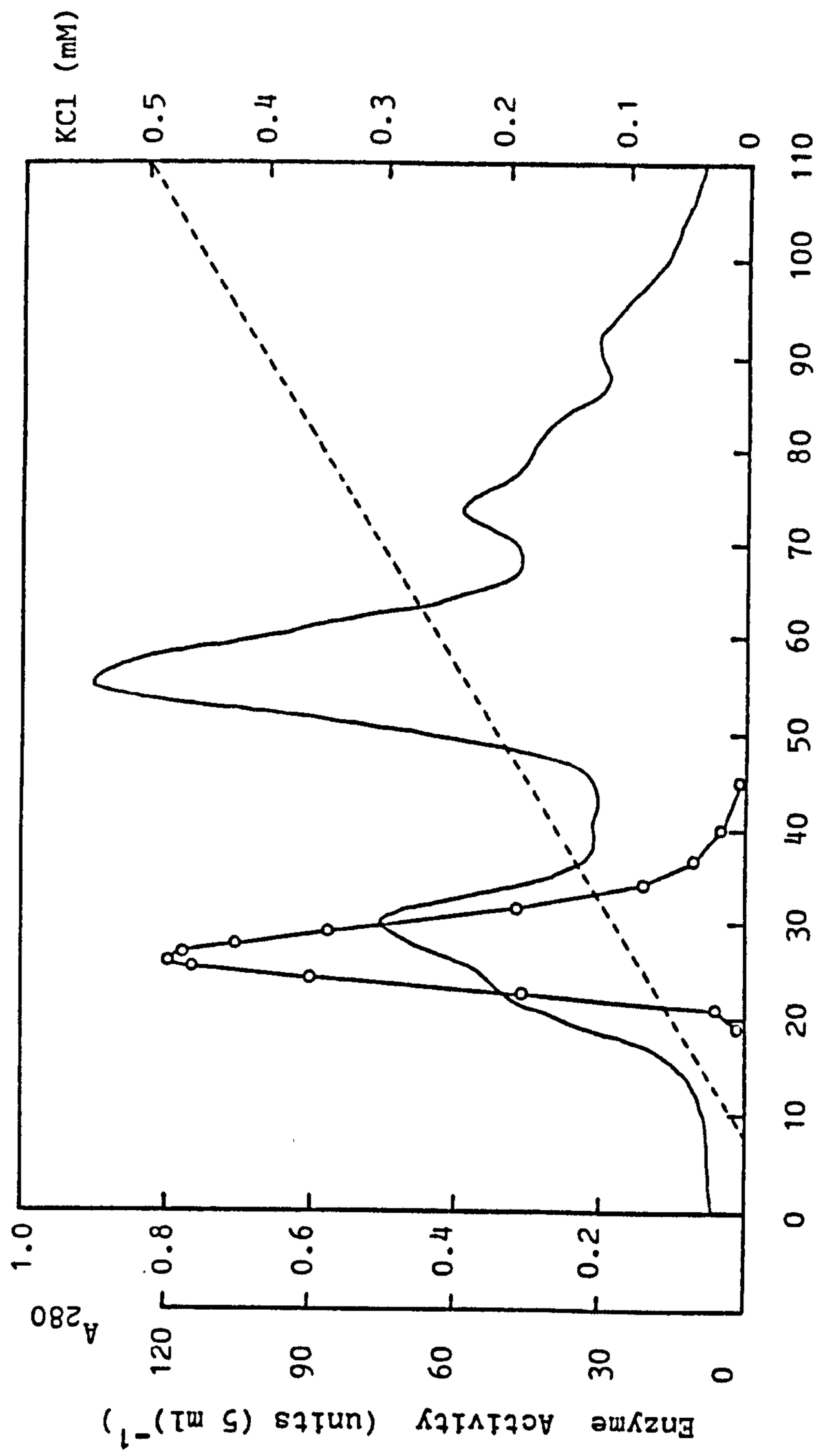
Following its initial partial purification, re-examination of the binding capacity of Red H-3B and Blue F3G-A for beta-ketothiolase revealed an increase to 25 and 18 units/ml Sepharose gel respectively. In each case the dye substitution on Sepharose CL-6B was 4 μmol/ml gel.

Optimisation of an elution protocol for beta-ketothiolase from each of the two conjugates indicated above generated a number of interesting results: Beta-ketothiolase bound to Red H-3B Sepharose was desorbed by addition of either ATP or CoASH to the equilibration buffer. In contrast, however, both of these cofactors, were ineffectual in desorbing the enzyme bound to Blue F3G-A Sepharose, even at a relatively high concentration of each (10 mM). Success in the elution of the enzyme from this conjugate, however, was effected by an increase in ionic strength of the equilibration buffer.

The choice of CoASH as the eluant of beta-ketothiolase from Red H-3B Sepharose in preference to ATP was based on differences in the selectivity of each cofactor in the desorption of this enzyme. CoASH dramatically resolved beta-ketothiolase from contaminating protein to produce a near homogeneous enzyme preparation, whereas a similar elution scheme using ATP resulted in the simultaneous elution of other proteins bound to the column.

Figure 5.3 Elution Profile of Beta-Ketothiolase from DEAE-Sepharose

The crude extract (1.5 g of protein) was applied to a column of DEAE-Sepharose (5 x 6.5 cm) equilibrated with phosphate buffer (20 mM, pH 6.5) containing dithiothreitol (1 mM). Elution was performed with a linear gradient of potassium chloride (0 - 0.5 M, in equilibration buffer, 400 ml) at a flow rate of 60 $ml.h^{-1}$. Fractions of 5 ml were collected. (O) Beta-ketothiolase activity; (---) KCl (M); (—) A_{280}.


KCl (mM)
0.5
0.4
0.3
0.2
0.1
0
110
100
90
80
70
60
50
40
30
20
10
0
Fraction Number
A_{280}
1.0
0.8
0.6
0.4
0.2
120
90
60
30
0
Enzyme Activity (units (5 ml)$^{-1}$)

5.3.2 ABSENCE OF ISOENZYMES OF BETA-KETOTHIOLASE

The presence of isoenzymes of beta-ketothiolase in S. cerevisiae (Kornblatt & Rudney, 1971a; 1971b), C. pasteurianum (Berndt & Schlegel, 1975) and most mammalian tissue (Middleton, 1973; Huth et al., 1974) prompted an evaluation of the phenomenon in M. trichosporium OB3b. Although unequivical evidence was not produced in this study, by isoelectric focussing for example, beta-ketothiolase nevertheless appeared to present as a single form in this organism. During elution of the enzyme from the ion-exchange column, activity was associated specifically with fractions containing the enzyme resolved to homogeneity in the second step of affinity chromatography; side fractions and the extract not bound to the column were devoid of beta-ketothiolase activity. Furthermore, gel filtration of the enzyme following affinity chromatography produced two peaks, the largest of which which corresponded exclusively to beta-ketothiolase activity. The small peak did not contain beta-ketothiolase activity.

5.3.3 STABILITY OF BETA-KETOTHIOLASE

The preparation of crude extracts of M. trichosporium OB3b by sonication without the prior addition of a sulphydryl reagent resulted in almost total inactivation of beta-ketothiolase. The inactivation, however, was not totally irreversible since 60 % of the initial activity of the enzyme was recovered following incubation of the extract with either dithiothreitol (1 mM), glutathionine (2 mM) or 2-mercaptoethanol (5 mM). Subsequently, dithiothreiotol was routinely

added to all buffers throughout the purification sequence. Presumably, this prevented inactivation of the enzyme through oxidation of a catalytically important sulphydryl group(s).

Long term storage of the beta-ketothiolase was achieved at -80°C in Tris-HCl buffer (50 mM, pH 7.5), containing dithiothreitol (1 mM) and glycerol (50 % v/v). By adopting this regime the enzyme lost less than 25 % activity following six months storage.

5.3.4 MOLECULAR WEIGHT OF BETA-KETOTHIOLASE

The molecular weight of purified beta-ketothiolase was determined by comparison of its elution volume, after chromatography on Sephacryl S-300, with that of known molecular weight standards. A molecular weight value of 140,000 daltons was obtained for the native protein by this process (Figure 5.4). The subunit molecular weight of 38,000 daltons was derived by SDS polyacrylamide gel electrophoresis (Figure 5.5), suggesting that the enzyme was probably tetrameric.

5.3.5 CATALYTIC PROPERTIES OF BETA-KETOTHIOLASE

The cleavage of acetoacetyl-CoA by beta-ketothiolase was measured at 303 nm in the presence of magnesium ions by following the decrease in the absorbance of the chelate (enolate) form of acetoacetyl-CoA. In solution acetoacetyl-CoA tautomerises between its keto and enol form, an equilibrium which is dependant on the concentration of H^+ ions (equation 5.5).

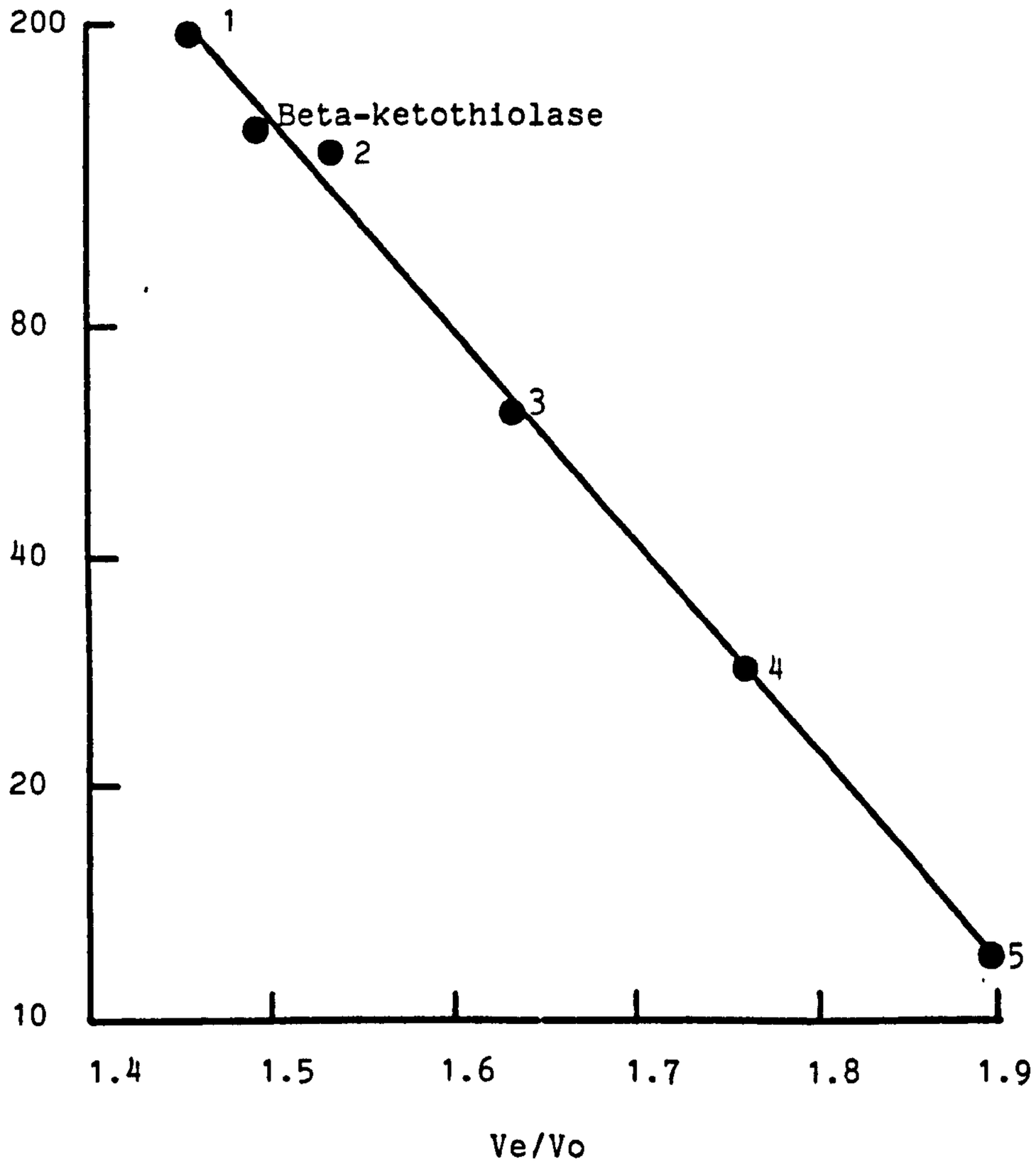


Figure 5.4 Molecular Weight Determination of Beta-Ketothiolase from M. trichosporium OB3b by Sephacryl S-300 Gel Filtration

The elution volume of beta-ketothiolase on a column of Sephacryl S-300 was compared to proteins of known molecular weight. 1. Beta-Amylase; 2. Alcohol dehydrogenase; 3. Albumin, Bovine; 4. Carbonic Anhydrase; 5. Cytochrome C. (see section 2.9 for further details).

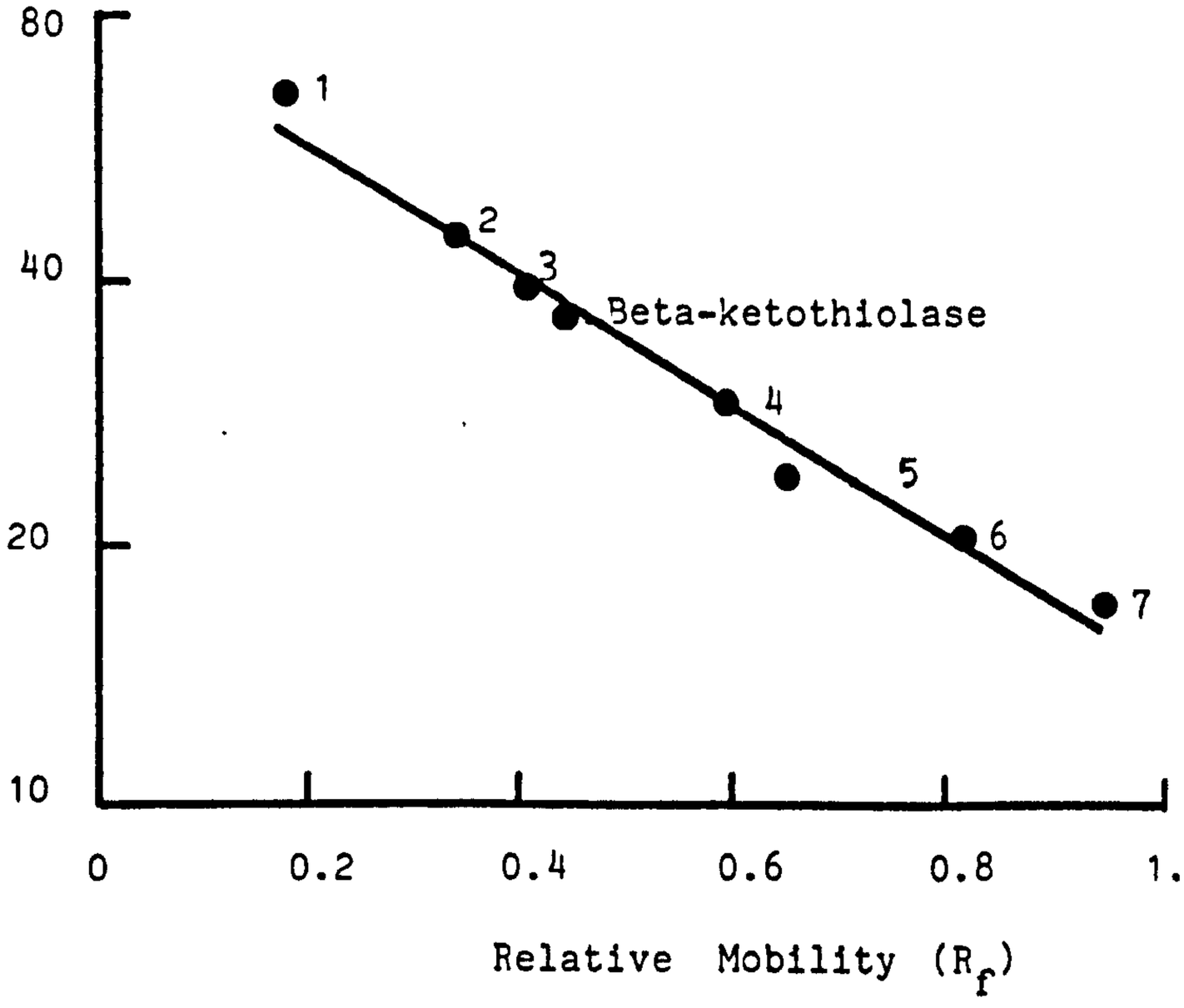
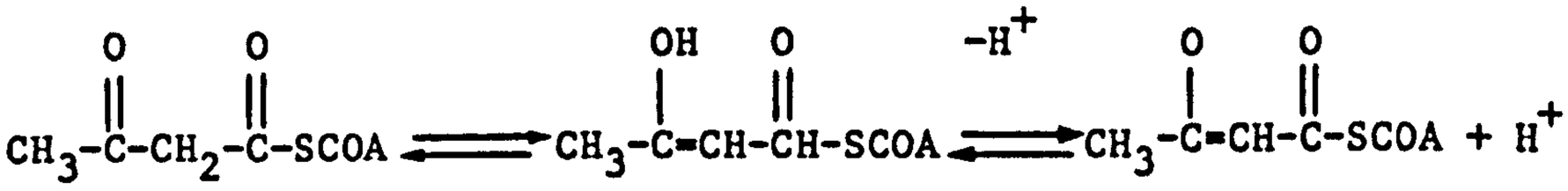


Figure 5.5 Determination of the Subunit Molecular Weight of Beta-Ketothiolase from M. trichosporium OB3b by SDS Polyacrylamide Gel Electrophoresis

The relative mobility of the dissociated enzyme on a 12 % polyacrylamide gel containing SDS, was compared to proteins of known molecular weight. 1. Albumin, Bovine; 2. Albumin, Egg; 3. Glyceraldehyde-3-phosphate dehydrogenase; 4. Carbonic Anhydrase; 5. Trypsinogen; 6. Trypsin Inhibitor, Soybean; 7. Alpha-Lactalbumin. (see section 2.10 for further details)



(Keto form) (Enol form) (Enolate form) ..(5.5)

At pH values above 7.5 magnesium ions interact with the enolate ion of acetoacetyl-CoA to form a chelate (1 magnesium ion per acetoacetyl-CoA ion; Stern, 1956). As shown in Figure 5.6 the molar extinction coefficient of acetoacetyl-CoA increases, such that at pH 9.6 it becomes constant due to the formation of the complete enolate form of acetoacetyl-CoA. Since the true substrate of beta-ketothiolase is assumed to be the keto-form of acetoacetyl-CoA (Stern, 1956; Middleton, 1974), the change in absorbance at 303 nm includes the reinstatement of the equilibrium between keto, enol and chelate forms of acetoacetyl-CoA during the cleavage reaction. A pH profile for the cleavage reaction, which accounts for changes in the molar extinction coefficient of acetoacetyl-CoA is presented in Figure 5.7.1. By this method a pH optimum of 8.35 was subsequently recorded for the enzyme reaction.

The condensation reaction catalysed by beta-ketothiolase exhibited a broad range of optimum activity (6.0 - 8.0) when assessed in both Tris-HCl and phosphate buffers, using the coupled optical test system described in section 5.2 (Figure 5.7.2).

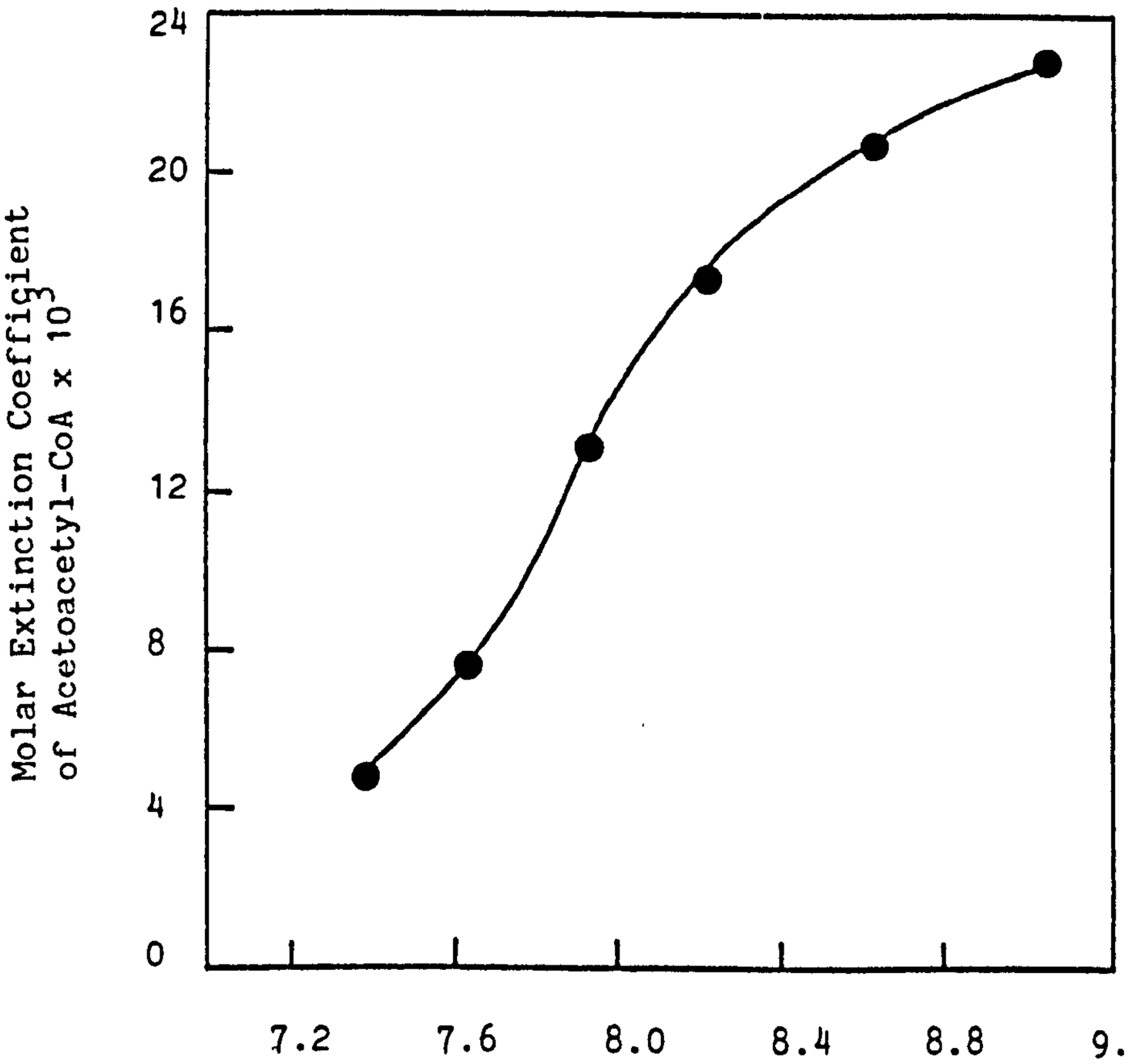


Figure 5.6 Dependance of the Molar Extinction Coefficient of Acetoacetyl-CoA on pH

The absorption (E_{303}) of acetoacetyl-CoA (30 μM) in Tris-HCl buffer (0.1 M) containing $MgCl_2$ (20 mM) was measured at the appropriate pH. The molar extinction coefficient of acetoacetyl-CoA was calculated from the Beer-Lambert relationship as described in section 5.2.3.

Figure 5.7 Effect of pH on the Activity of Beta-Ketothiolase from M. trichosporium

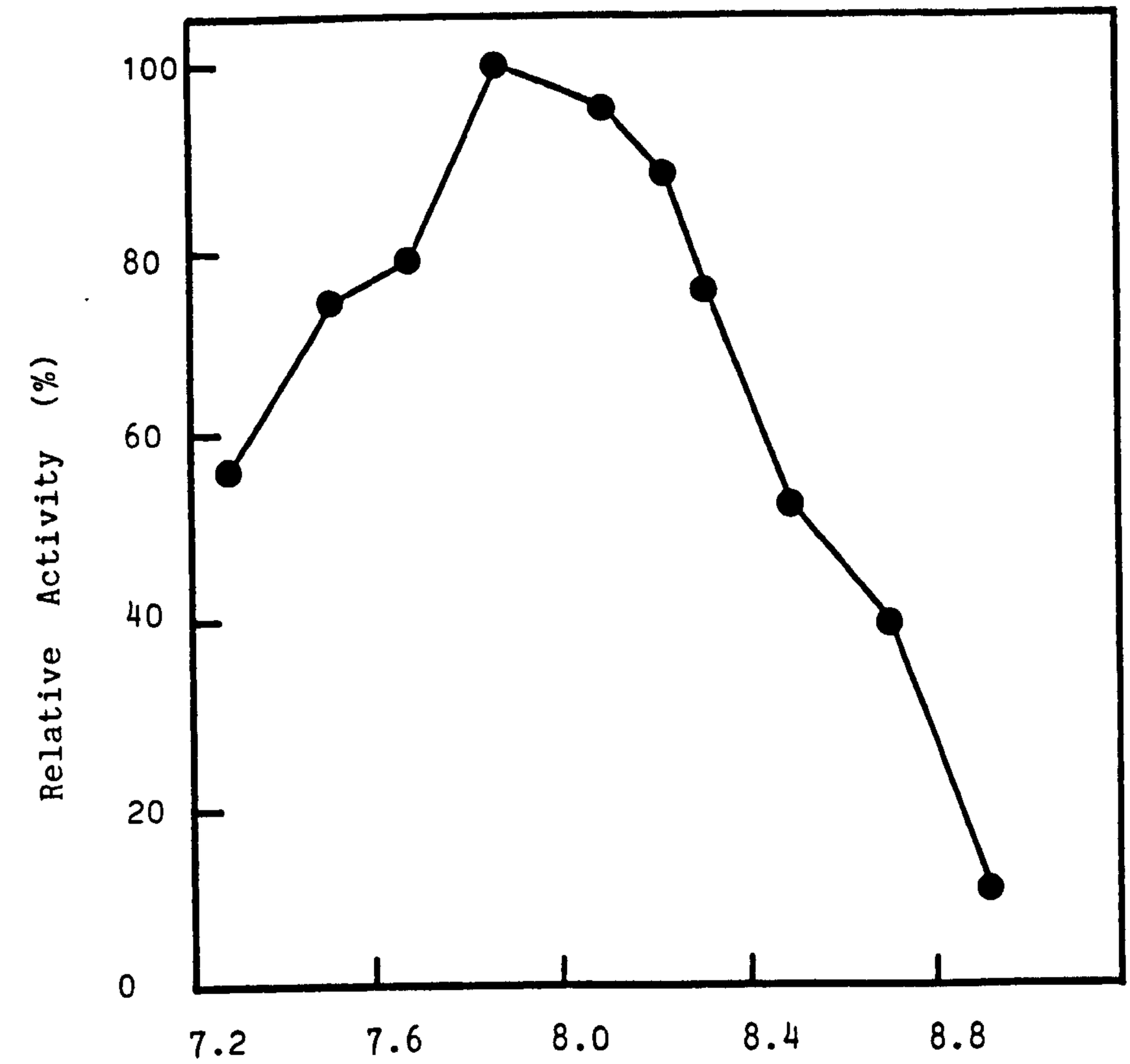
The cleavage and condensation reactions catalysed by beta-ketothiolase were measured at the appropriate pH by the methods described in section 5.2.2. The relative activities obtained in (1) represent a correction for the pH dependant change in the molar extinction coefficient of acetoacetyl-CoA. Buffers used in this study were as follows:

(O) Phosphate buffer (100 mM); (●) Tris-HCl buffer (100 mM).

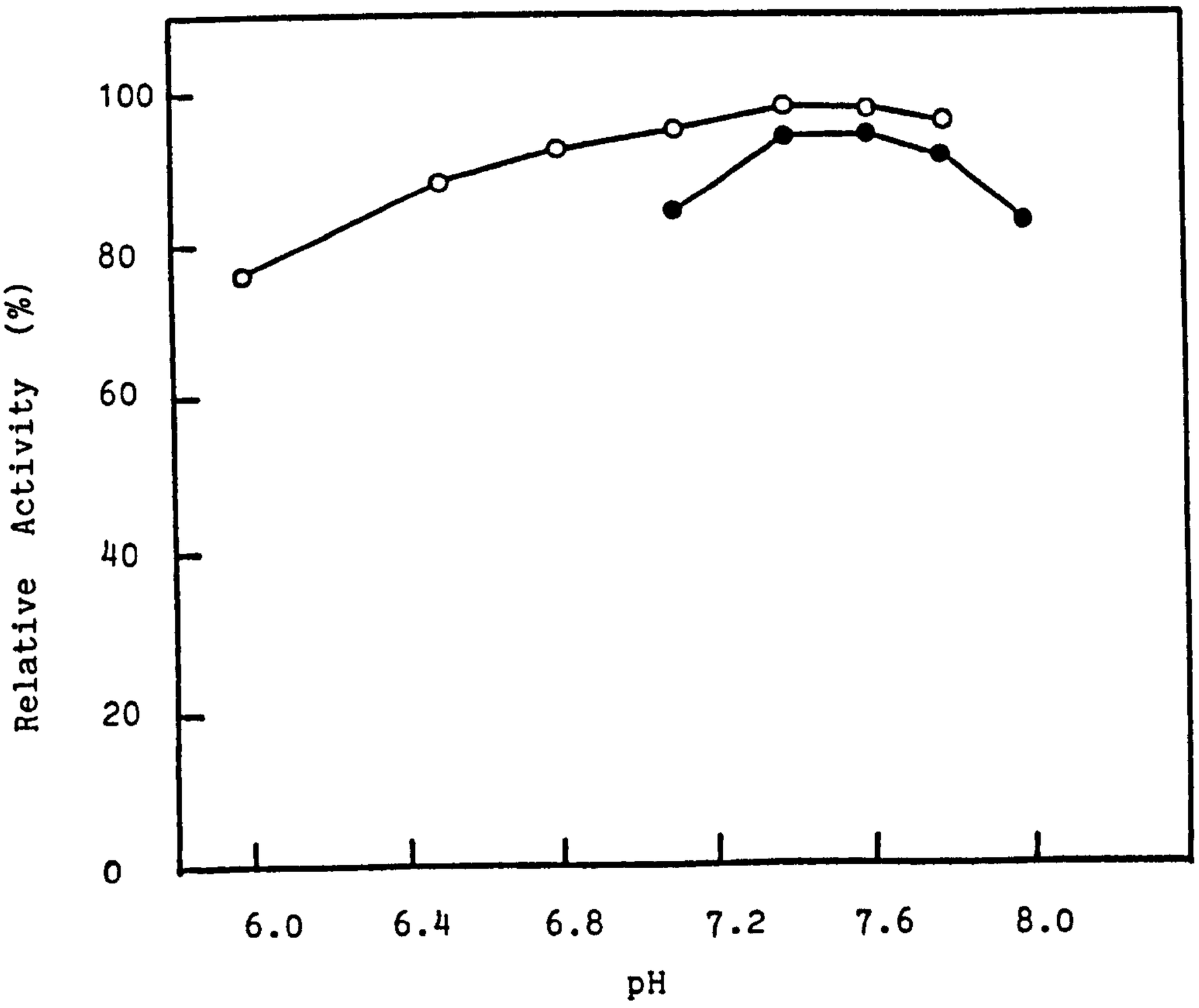
(1) Acetoacetyl-CoA cleavage.

(2) Condensation of acetyl-CoA.

(1)


Relative Activity (%)
100
80
60
40
20
0
7.2
7.6
8.0
8.4
8.8
pH

(2)


Relative Activity (%)
100
80
60
40
20
0
6.0
6.4
6.8
7.2
7.6
8.0
pH

5.3.6 KINETIC PROPERTIES OF BETA-KETOTHIOLASE

In order to assess the possible in vivo regulation of beta-ketothiolase from M. trichosporium OB3b, both in terms of its role in PHB synthesis and during PHB mobilisation, a detailed kinetic study on the purified enzyme was initiated.

5.3.6.1 KINETICS OF THE CONDENDSATION REACTION

In the direction of PHB synthesis beta-ketothiolase catalyses the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA and CoASH. During this study the reaction rate was measured by a coupled optical test system in which the product of the reaction, acetoacetyl-CoA, was reduced by the auxillary enzyme 3-hydroxyacyl-CoA dehydrogenase in the presence of NADH. The auxillary enzyme is ideally suited to its role in the assay system by virtue of its affinity for acetoacetyl-CoA (K_m = 5 x 10^{-5} M; Bergmeyer, 1970) and an equilibrium constant (K_{eq} = 2.17 x 10^{-10}; Lynen & Wieland, 1955) which strongly favours the formation of D(-)-3-hydroxybutyryl-CoA. Furthermore, the enzyme was not inhibited by either the substrate (acetyl-CoA) or the products (acetoacetyl-CoA or CoASH) of the condensation reaction.

Kinetic studies on the condensation reaction were conducted by measuring the reaction rate at variable concentrations of acetyl-CoA (0.5 - 4.0 mM). Under these conditions, in the absence of products, the enzyme exhibited normal Michaelis-Menten kinetics. The Lineweaver-Burk transformation (1934) of this data (Figure 5.8)

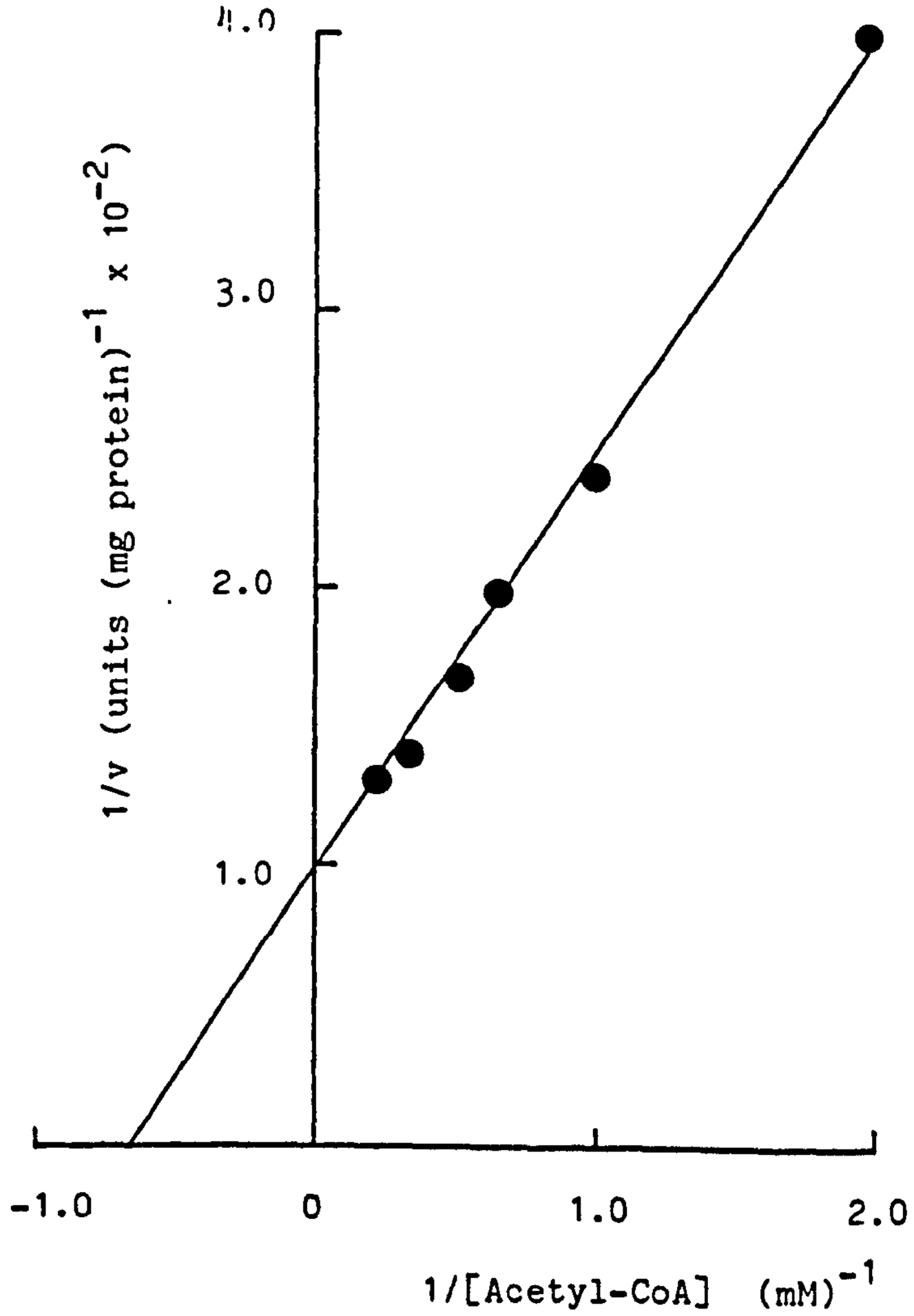


Figure 5.8 Lineweaver-Burk Transformation of Kinetic Data for the Condensation of Acetyl-CoA Catalysed by Beta-Ketothiolase at Different Initial Levels of Acetyl-CoA

Cuvettes contained in a total reaction volume of 1 ml: Tris-HCl buffer (100 mM, pH 7.5); 3-hydroxyacyl-CoA dehydrogenase (1 unit); NADH (0.15 mM); purified enzyme preparation (10.7 µg of protein). Acetyl-CoA concentration was varied as indicated.

produced a straight line, from which an apparent K_m for acetyl-CoA of 1.56×10^{-3} M was obtained, together with a V_{max} of 120 units (mg protein)$^{-1}$.

Since CoASH is known to be a vigorous inhibitor of all the bacterial beta-ketothiolases studied to date, further kinetic studies on the methanotrophic enzyme were conducted by varying the acetyl-CoA concentration at several fixed levels of CoASH (0.075 - 0.3 mM). This study had two objectives. Firstly, it would reveal information on the kinetic regulation of this enzyme, from which inferences regarding a possible control mechanism for PHB accumulation could be made. Additionally, the study would also provide information on the enzyme reaction mechanism (Cleland, 1970).

In the presence of CoASH the rate of the condensation reaction decreased and the substrate (acetyl-CoA) saturation curves became increasingly sigmoidal (Figure 5.9.1). When this data was presented as a Lineweaver-Burk plot (Figure 5.9.2), non-linear Michaelis-Menten kinetics were observed. However, it appears that each curve intersects at one point on the ordinate axis indicating that V_{max} is achieved at high concentrations of acetyl-CoA even in the presence of increasing concentrations of CoASH. Essentially, the data describes a non-linear competitive inhibition of beta-ketothiolase by CoASH with respect to acetyl-CoA concentration. The non-linearity of the double reciprical plot precluded an estimation of the K_i for CoASH. However, it is clearly an effective inhibitor of the condensation reaction.

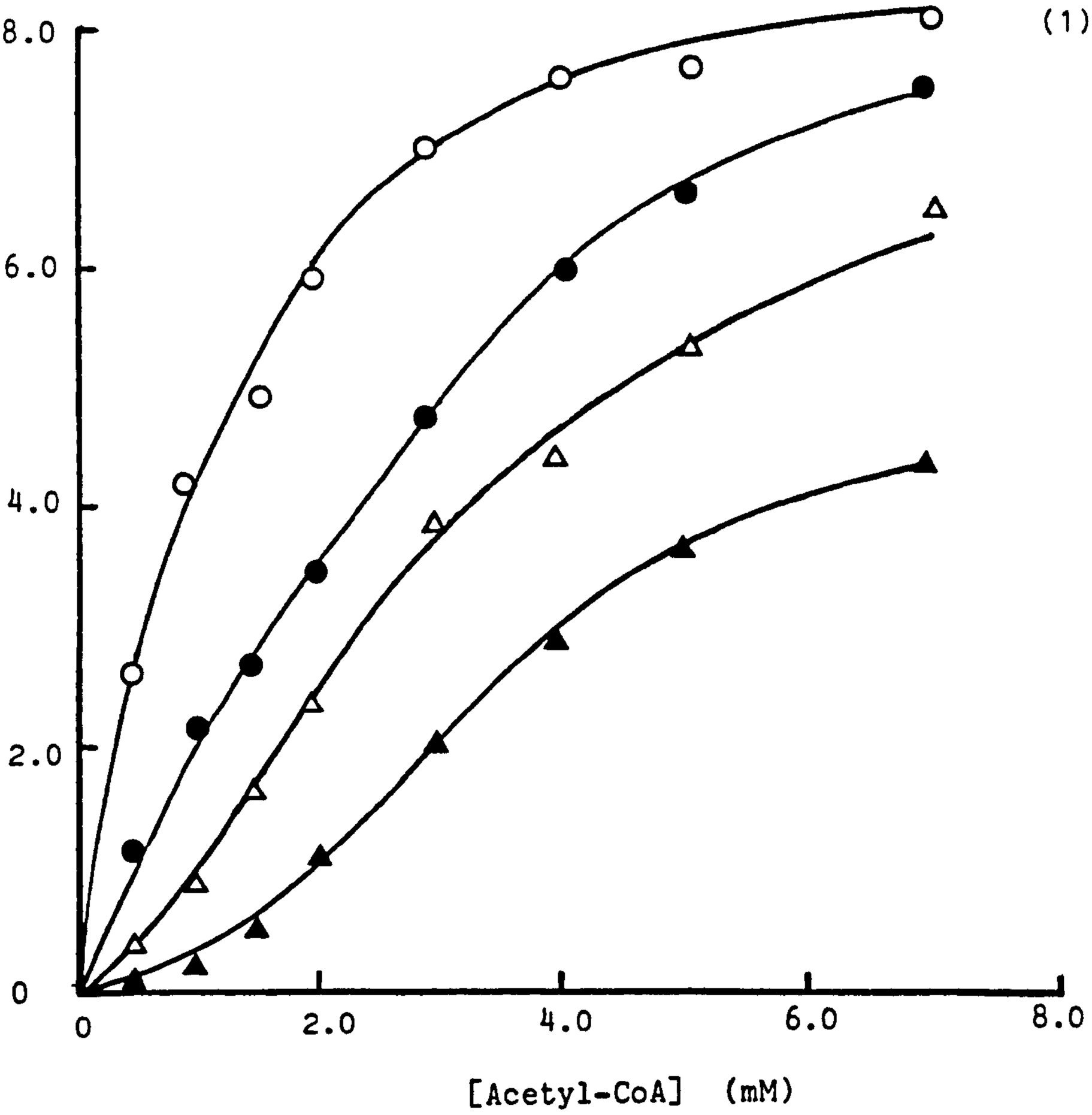
Figure 5.9 Substrate (Acetyl-CoA) Saturation Curves of Beta-Ketothiolase in the Absence and Presence of CoASH

Cuvettes contained in a total reaction volume of 1 ml: Tris-HCl buffer (100 mM, pH 7.5); 3-hydroxyacyl-CoA dehydrogenase (1 unit); NADH (0.15 mM); acetyl-CoA (0.5 - 4.0 mM) and purified enzyme preparation (10.7 µg of protein). Concentration of CoASH (mM): (○) control (no CoASH); (▲) 0.3; (△) 0.15; (●) 0.075.

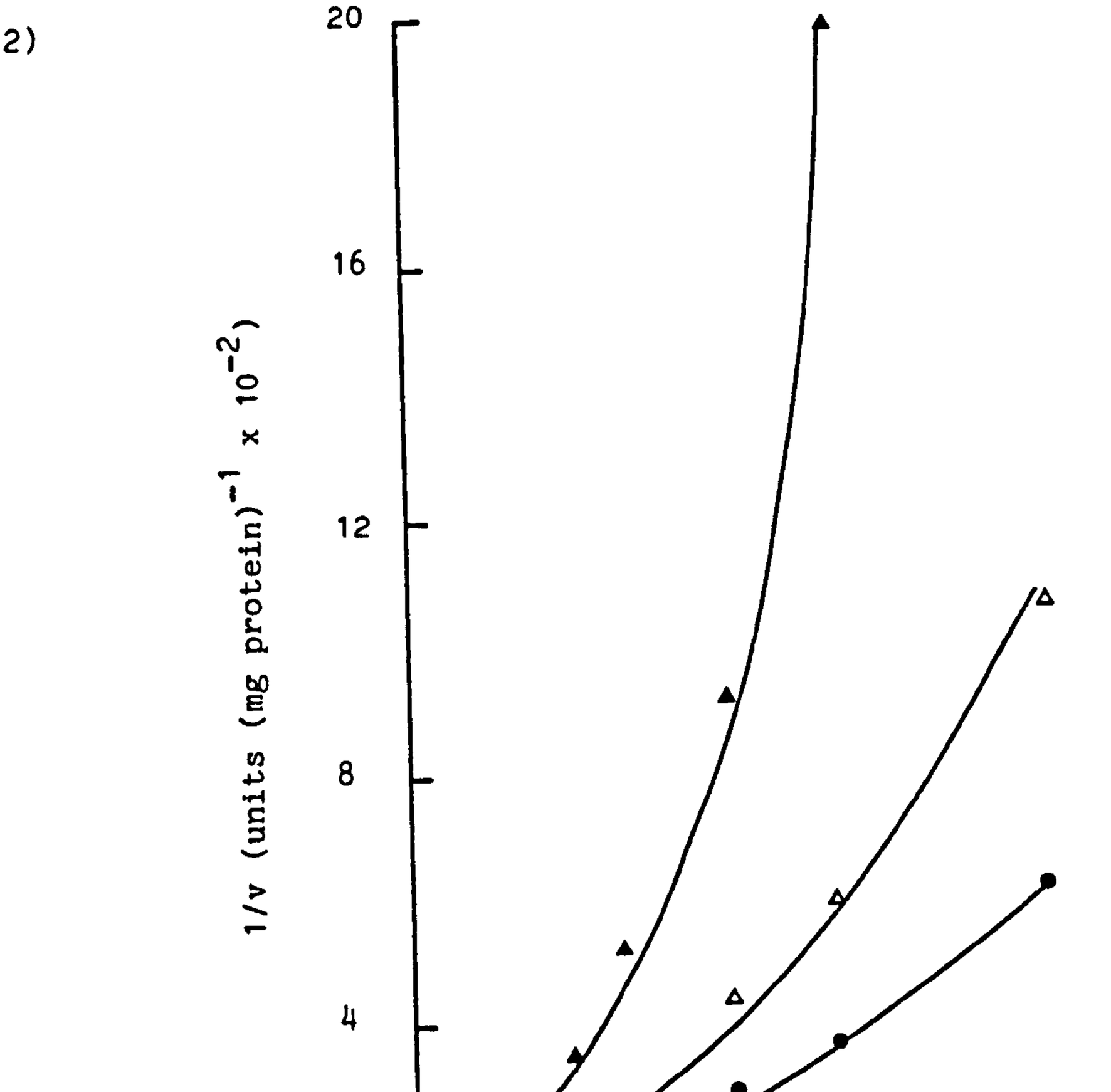
(1) Substrate saturation curves.

(2) Lineweaver-Burk plot of data obtaind from (1).

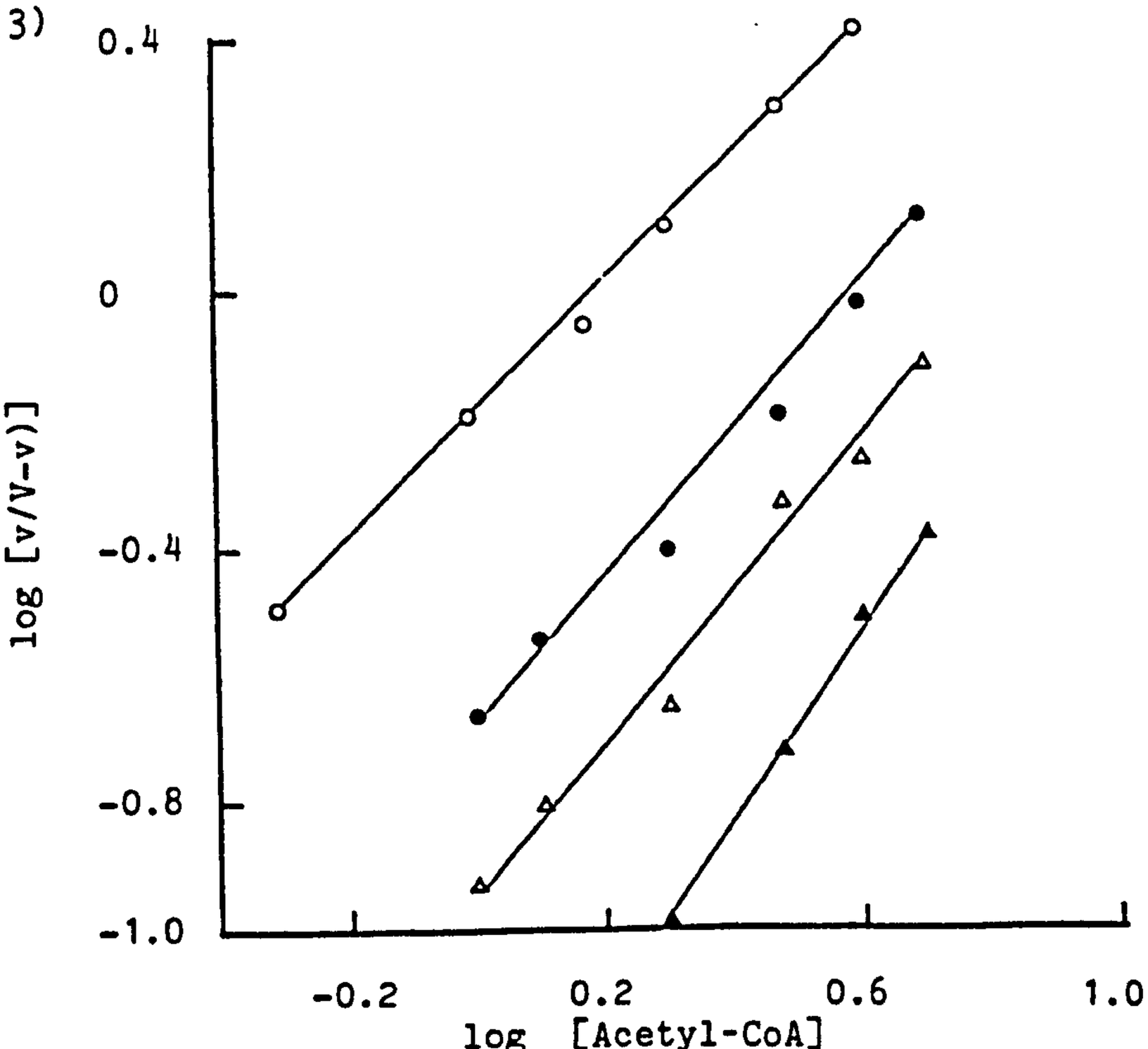
(3) Hill plot.


(1)
Enzyme Activity (units (mg protein)$^{-1}$ x 10^1)
8.0
6.0
4.0
2.0
0
0
2.0
4.0
6.0
8.0
[Acetyl-CoA] (mM)

(2)


20
16
12
8
4
1/v (units (mg protein)$^{-1}$ x 10^{-2})
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1.0
1/[Acetyl-CoA] (mM)$^{-1}$

(3)


0.4
0
-0.4
-0.8
-1.0
log [v/V-v)]
-0.2
0.2
0.6
1.0
log [Acetyl-CoA]

Although non-linear kinetics are sometimes attributable to a co-operative interaction between the enzyme and its substrates or products (Engel, 1981), in this case it is probably a function of the reaction mechanism; this receives a detailed appraisal in the discussion (Section 5.4). The increase in the slope (n_h) of the Hill plot with increasing CoASH concentration (Figure 5.9.3) is therefore interpreted as a measurement of the non-linearity of the reaction and not necessarily as an indicator of positive co-operativity.

5.3.6.2 KINETICS OF THE CLEAVAGE REACTION

The cleavage (thiolysis) of acetoacetyl-CoA by beta-ketothiolase results in the formation of two molecules of acetyl-CoA. The kinetics of thiolysis as a two substrate reaction were determined by varying the concentration of the first substrate and then of the second one.

Saturation curves for CoASH as a substrate of the cleavage reaction were produced for five fixed concentrations of acetoacetyl-CoA (2 to 12 µM) (Figure 5.10.1). At low levels of acetoacetyl-CoA an inhibition of enzyme activity by CoASH was observed, which was competitive with respect to acetoacetyl-CoA. An increase in the concentration of acetoacetyl-CoA had the effect of shifting inhibition by CoASH to a higher concentration. Presentation of the uninhibited data as a Lineweaver-Burk plot (Figure 5.10.2) gave a series of parallel lines which are indicative of a ping pong reaction mechanism (Cleland, 1967, 1970) as opposed to a sequential mechanism for the cleavage reaction.

Figure 6.1 SDS Polyacrylamide Gel Electrophoresis of Acetoacetyl-CoA Reductase from M. trichosporium OB3b

Samples from each step in the purification sequence were electrophoresed on 12 % gels by the method of Laemmli (1970) (section 2.10). Proteins were stained with Coomassie brilliant blue.

Lane 1, molecular weight markers (25 µg);

Lane 2, Crude extract (20 µg);

Lane 3, Blue MX-2R eluate (20 µg);

Lane 4, Blue MX-R eluate (10 µg).

STEP	VOLUME (ml)	PROTEIN (mg/ml)	TOTAL PROTEIN (mg)	TOTAL ACTIVITY (units)*	SPECIFIC ACTIVITY (µmol/ min/mg)	PURIFICATION FACTOR (fold)	YIELD (%)
Cell free extract	220	4.13	907.5	194	0.21	1	100
Procion Blue MX-2R	97	0.19	18.43	164	8.89	41.5	84.5
Procion Blue MX-R	24	0.03	0.70	127	39.60	185	66

* 1 unit = 1 µmole NADPH oxidised $min.^{-1}$ at $25^{o}C$

Table 6.1 Purification of Acetoacetyl-CoA Reductase from M. trichosporium OB3b

The table shows a summary of a typical purification from 50g of frozen cell paste. Experimental details are described in section 6.2.1.

6.3 RESULTS

6.3.1 PURIFICATION OF ACETOACETYL-COA REDUCTASE FROM M. TRICHOSPORIUM OB3B

The purification scheme described in this section, and summarised in Table 6.1, resulted in the resolution of acetoacetyl-CoA reductase from M. trichosporium OB3b to near homogeneity as indicated by SDS polyacrylamide gel electrophoresis (Figure 6.1). The procedure contained two steps of triazine dye affinity chromatography. The first step utilised a relatively non-specific ionic elution of acetoacetyl-CoA reductase from Procion Blue MX-2R Sepharose, whereas the second step incorporated a highly selective cofactor (NADPH) elution of the enzyme from Procion Blue MX-R Sepharose. By adopting this purification protocol the final yield of acetoacetyl-CoA reductase from M. trichosporium OB3b was in excess of 60 % of the total initial activity detected in the crude extract.

In view of the dependancy of acetoacetyl-CoA reductase on NADPH for catalytic activity, information regarding the interaction of triazine dyes with NADPH-dependant enzymes (Watson et al., 1978; Dean & Watson, 1978; Lowe, 1979) served to highlight the potential of these ligands in the possible purification of this enzyme. Consequently, a range of dye-ligands, immobilised on Sepharose CL-6B, were screened for their suitability to bind acetoacetyl-CoA reductase from the crude extract of M. trichosporium OB3b. The dyes tested included Red H-3B,

this assay, one unit of enzyme was defined as the amount of enzyme that, under the conditions of the assay, catalysed the cleavage of 1 µmole of acetoacetyl-CoA to form two molecules of acetyl-CoA in 1 min. at 25°C.

6.2.3 DETERMINATION OF THE EQUILIBRIUM CONSTANT OF ACETOACETYL-COA REDUCTASE FROM M. TRICHOSPORIUM OB3B

The reaction mixture contained Tris-HCl buffer (100 mM, pH 7.5 or pH 8.0), acetoacetyl-CoA (30 µM), NADPH (0.05 mM) and purified enzyme (2.9 µg of protein) in a total reaction volume of 1 ml. The reaction was initiated by the addition of acetoacetyl-CoA and the extinction recorded continuously until equilibrium was reached. Final concentrations of each component at equilibrium was calculated from the change in extinction at 340 nm.

was washed with equilibration buffer (100 ml) and the enzyme eluted in an upwards direction with NADPH (0.5 mM) in the same buffer (10 ml). Following application of NADPH, the column was washed with equilibration buffer (20 ml) until elution of the enzyme was complete. All acetoacetyl-CoA reductase activity was collected, dialysed by diafiltration against ten volumes of buffer, and used in subsequent analysis.

6.2.2 ENZYME ASSAYS

Acetoacetyl-CoA reductase activity was assayed by monitoring either (A) NADPH disappearance or (B) the breakdown of the magnesium enolate form of acetoacetyl-CoA.

A. NADPH Disappearance. Cuvettes contained, in a total reaction volume of 1 ml: Tris-HCl buffer (100 mM, pH 8.8); acetoacetyl-CoA (30 μM) and NADPH (0.15 mM). The reaction was initiated by the addition of acetoacetyl-CoA and followed by measuring the decrease in absorbance at 340 nm, due to the disappearance of NADPH. Using this assay, one unit of enzyme was defined as the amount of enzyme that, under the conditions of the assay, catalysed the oxidation of 1 μmole of NADPH in 1 min. at 25°C.

B. Enolate Breakdown. Cuvettes contained, in a total reaction volume of 1 ml: Tris-HCl buffer (100 mM, 20 mM $MgCl_2$, pH 8.2); acetoacetyl-CoA (30 μM) and NADPH (0.15 mM). The reaction rate was measured by monitoring the decrease in absorbance, at 303 nm, due to the breakdown of the magnesium enolate form of acetoacetyl-CoA. Using

6.2 EXPERIMENTAL

6.2.1 PROTOCOL FOR THE PURIFICATION OF ACETOACETYL-COA REDUCTASE FROM M. TRICHOSPORIUM OB3B

Frozen cells (50 g) were thawed and resuspended in potassium phosphate buffer (20 mM, pH 7.0 containing dithiothreitol (1 mM), 200 ml) at room temperature. The cell-free extract was prepared as described previously (Section 2.5), with the exception that the final pH was adjusted to 7.0. Potassium phosphate buffer (20 mM, pH 7.0, containing 1 mM dithiothreitol) was used in both steps of the purification sequence. All operations were carried out at $4^{o}C$ where possible.

STEP 1: Chromatography on Procion Blue MX-2R Sepharose

The cell free extract was applied to a column of Procion Blue MX-2R (2.5 x 8.5 cm) at a flow rate of 60 $ml.h^{-1}$. The column was washed with equilibration buffer (200 ml) to remove unbound proteins and the enzyme eluted with a linear gradient (400 ml) of potassium chloride (0 - 0.5 M) in phosphate buffer. Active fractions (5 ml) were pooled and dialysed by diafiltration (Amicon hollow fibre cartridge, 30,000 mol. wt. cut off) against phosphate buffer (500 ml).

STEP 2: Chromatography on Procion Blue MX-R Sepharose

The dialysate from the previous step was applied to a column of Procion Blue MX-R (1.5 x 8 cm) at a flow rate of 90 $ml.h^{-1}$. The column

enzyme, which was thought to participate in PHB synthesis in this organism, was purified 150-fold and was shown to have a K_m of 8.3 x 10^{-6} M for acetoacetyl-CoA and 21 x 10^{-6} M for NADP. Unlike the enzyme from A. beijerinkii acetoacetyl-CoA reductase from this organism was not inducible during polymer synthesis.

Although Z. ramigera I-16-M contained enoyl-CoA hydratase activity, the presence of a pathway of the type described in R. rubrum (Moskowitz & Merrick, 1969) was dismissed following radioisotope-tracer studies. This indicated that L(+)-hydroxybutyryl-CoA was not converted to its D(-)-isomer, the substrate for PHB synthase, through the action of enoyl-CoA hydratase, but that the L(+)-isomer could be oxidised by an NAD-linked dehydrogenase to acetoacetyl-CoA, and then reduced to the D(-)-isomer by an NADPH-dependent reductase.

To complete the study on the soluble enzymes associated with PHB metabolism in M. trichosporium OB3b, it was essential to determine the pathway leading to D(-)-3-hydroxybutyryl-CoA formation. As eluded to in the previous chapter (section 5.4), a detailed analysis of acetoacetyl-CoA metabolism in this organism was necessitated in order to postulate a possible control mechanism for PHB synthesis. This chapter describes the techniques required to purify the enzyme associated with acetoacetyl-CoA metabolism in this organism and further to highlight its role in the regulation of PHB synthesis.

NADPH-specific, notable exceptions include the enzyme from B. cereus which was NADH-specific (Kominek & Halvorson, 1965), and that from a species of Hydrogenomonas which had a similar cofactor dependancy in addition to NADPH-linked activity (Schindler, 1964). In the latter case NADPH was shown to support 50 % of the initial activity obtained with NADH. In retrospect, it is probably worth noting that in neither case was the immediate product of the reaction identified as D(-)- or L(+)-3-hydroxybutyryl-CoA.

A detailed study on acetoacetyl-CoA reductase from A. beijerinkii revealed that activity was induced five-fold during batch growth prior to the onset of the stationary phase and the accumulation of PHB (Ritchie et al., 1971). The reduction reaction product was identified as D(-)-3-hydroxybutyryl-CoA and NADPH was the preferred coenzyme, NADH giving one-fifth the reaction rate under the same assay conditions. The K_m of the enzyme for acetoacetyl-CoA was estimated in the range of 2.9 - 21.0 x 10^{-6} M. Subsequent studies on this enzyme (Senior & Dawes, 1973), using fluorescent detection of NADPH reduction, estimated a K_m value for acetoacetyl-CoA in the range 1-2 x 10^{-6} M. This study also revealed that a concentration of acetoacetyl-CoA in excess of 10 μM gave rise to a significant inhibition of reductase activity; no further activators or inhibitors of this enzyme were indicated.

Z. ramigera I-16-M was found to contain two stereospecific acetoacetyl-CoA reductases, one of which was NADP-linked and D(-)-3-hydroxybutyryl-CoA specific while the other was NAD-linked and specific for the L(+)-isomer (Saito et al., 1977). The NADP-linked

Stern et al. (1955) demonstrated L(+)-3-hydroxybutyryl-CoA dehydrogenase activity in extracts of R. rubrum, and also reported that such extracts catalysed the interconversion of the D(-)- and L(+)-isomers of 3-hydroxybutyryl-CoA; the mechanism of the racemisation was not studied. Later, studies on the same organism (Moskowitz & Merrick, 1969) revealed that the racemisation was catalysed by two highly specific enoyl-CoA hydratases. Enoyl-CoA hydratase (D) gave rise to the D(-)-isomer while the action of enoyl-CoA hydratase (L) resulted in the formation of L(+)-3-hydroxybutyryl-CoA. On the basis of this information the pathway leading to PHB synthesis from acetoacetyl-CoA was subsequently proposed (see equation 6.1).

Further analysis of purified enoyl-CoA hydratase (D), highlighted its broad specificity for various crotonyl thioesters; these included crotonyl pantetheine (K_m = 1.18 x 10^{-4} M) and crotonyl acyl carrier protein (K_m = 2.63 x 10^{-5} M). The product of the crotonyl acyl carrier protein hydration, however, did not serve as a substrate for the PHB synthase of this organism, suggesting that the acyl carrier protein thioester does not play a significant role in PHB synthesis.

Acetoacetyl-CoA reductase activity has been reported in all other PHB producing microorganisms studied to date. These include R. spheroides (Carr & Lascelles, 1961); A. eutropha H16 (Schindler, 1964); B. cereus (Kominek and Halvorson, 1965); A. beijerinkii (Ritchie et al., 1971); Z. ramigera I-16-M (Saito et al., 1977) and in the facultative methylotroph, Ps. AM1 (Taylor & Anthony, 1976). Although most sources of acetoacetyl-CoA reductase appear to be

6.1 INTRODUCTION

The formation of D(-)-3-hydroxybutyryl-CoA in bacteria, prior to its polymerisation to form PHB occurs through two distinctly different metabolic routes:

(a) In R. rubrum (Moskowitz & Merrick, 1969), acetoacetyl-CoA is first reduced to L(+)-3-hydroxybutyryl-CoA by an NAD-linked dehydrogenase, and then converted successively into D(-)-3-hydroxybutyryl-CoA through the action of two stereospecific enoyl-CoA dehydratases (equation (6.1)).

$$\text{Acetoacetyl-CoA} + \text{NAD(P)H} + H^{+} \rightleftharpoons \text{L(+)-3-Hydroxybutyryl-CoA} + \text{NADP}$$

$$\text{L(+)-3-Hydroxybutyryl-CoA} \rightleftharpoons \text{Crotonyl-CoA} + H_2O$$

$$\text{Crotonyl-CoA} + H_2O \rightleftharpoons \text{D(-)-3-Hydroxybutyryl-CoA} \quad \ldots(6.1)$$

(b) In contrast, the majority of bacteria studied to date, utilise an acetoacetyl-CoA reductase (D(-)-3-hydroxybutyryl-CoA-NADP oxidoreductase, EC 1.1.1.36), which catalyses the direct formation of D(-)-3-hydroxybutyryl-CoA from acetoacetyl-CoA (Equation 6.2)).

$$\text{Acetoacetyl-CoA} + \text{NADPH} + H^{+} \rightleftharpoons \text{D(-)-3-Hydroxybutyryl-CoA} + \text{NADP} \quad (6.2)$$

C H A P T E R S I X

THE PURIFICATION AND PROPERTIES OF ACETOACETYL-COA REDUCTASE FROM M. TRICHOSPORIUM OB3B

beta-ketothiolase forms part of a concerted inhibition of PHB mobilisation it is difficult to envisage its importance in this context. On the other hand, regulation of acetoacetyl-CoA cleavage by CoASH might play a part in preventing a futile cycle of acetyl-CoA production following its condensation to form acetoacetyl-CoA. This, however, is also dependant upon the kinetics of the enzyme metabolising acetoacetyl-CoA in the direction of PHB synthesis. This is investigated in the next chapter.

The conclusions drawn from previous chapters on 3-HBD (Chapter 3) and acetoacetyl-CoA synthetase (Chapter 4) suggested that an alternative route for acetoacetate metabolism might exist in this organism. Although the kinetic data in this study does not preclude this possibility it did suggest that beta-ketothiolase from this organism is "kinetically designed" to catalyse both the cleavage and the formation of acetoacetyl-CoA. Beta-ketothiolases operating exclusively in a synthetic mode are characteristically susceptable to a double competitive inhibition by both its substrates (Berndt & Schlegel, 1975; Kornblatt & Rudney, 1971a). This, however, was not the case in M. trichosporium OB3b. Nevertheless, acetoacetate metabolism in this organism certainly requires more detailed analysis before one can categorically preclude the operation of a split pathway operating at the level of acetoacetate.

synthase which was not inhibited by physiological concentrations of a range of metabolites (Colby & Zatman, 1975). In contrast, PHB synthesis in both A. eutrophus H16 and A. beijerinkii was thought to reflect inhibition of citrate synthase by NADH, resulting in a high acetyl-CoA/CoASH ratio. In the absence of this type of control in M. trichosporium OB3B one can postulate that PHB synthesis might occur as a result of acetyl-CoA overspill metabolism. Certainly the K_m of beta-ketothiolase for acetyl-CoA is indicative of optimum activity of this enzyme at a relatively high concentration of the metabolite (Cleland, 1970).

Since the activity of the TCA cycle in this organism is low (Colby & Zatman, 1975) one could envisage that, under conditions where acetyl-CoA is surplus to the requirements of the cell, redirection via PHB synthesis precludes it accumulation. However, these possibilities remain speculative in the absence of in vivo metabolite measurement of acetyl-CoA and CoASH prior to PHB synthesis. It is therefore difficult to rationalise the importance of the ratio of these metabolites to the control of beta-ketothiolase in vivo; this issue is investigated in chapter seven.

From the kinetic and regulatory data obtained for beta-ketothiolase operating in the direction of acetoacetyl-CoA cleavage it appears that this reaction is regulated through the acetoacetyl-CoA/CoASH ratio. The importance of this information in the context of PHB mobilisation is unclear. Classically, regulation of a metabolic sequence occurs after the first step in the pathway or at branch points in the metabolic sequence. Unless CoASH inhibition of

at fixed CoASH concentrations and when CoASH was varied at fixed acetoacetyl-CoA concentrations. Under the conditions of this investigation the methanotrophic enzyme was shown to be susceptible to inhibition by CoASH, which was competitive with respect to acetoacetyl-CoA. This suggested that both substrates were capable of binding to the same enzyme form, which, in the context of the reaction mechanism described previously for this enzyme (equation 5.6), would indicate that CoASH was capable of forming an abortive complex with the free enzyme. The overall effect would be to diminish the effective concentration of enzyme available to perform the condensation reaction.

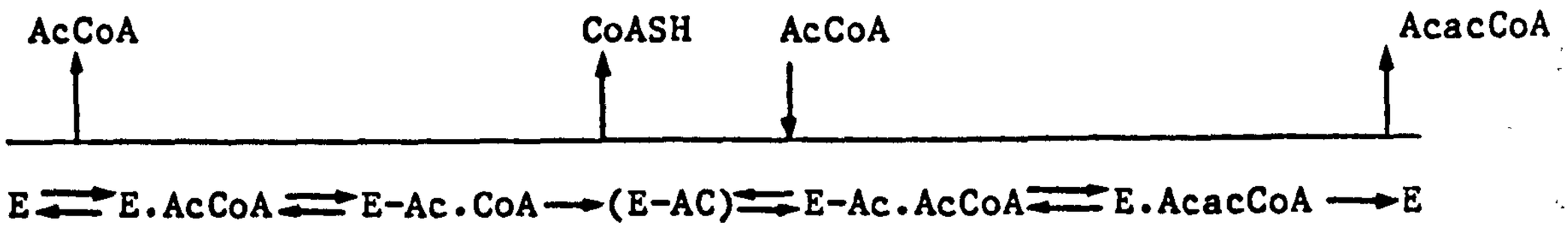
5.4.2 SIGNIFICANCE OF THE KINETIC AND REGULATORY PROPERTIES OF BETA-KETOTHIOLASE

In the direction of acetoacetyl-CoA formation, the kinetic properties of beta-ketothiolase from M. trichosporium OB3b suggested that the regulation of PHB synthesis in this organism was mediated through the in vivo acetyl-CoA/CoASH ratio. Similar results were obtained with the beta-ketothiolase from other PHB producing bacteria including A. eutrophus H16 (Oeding & Schlegel, 1973) and A. beijerinkii (Senior & Dawes, 1973). This led to the general conclusion that the activity of the TCA cycle, or more specifically citrate synthase, was the major contributary factor in determining the acetyl-CoA/CoASH ratio and hence the regulation of PHB synthesis. However, in retrospect this argument is not necessarily applicable to the organism in this study. M. trichosporium OB3b, together with a number of methanotrophs, possesses a low molecular weight citrate

presence of CoASH. Subsequently, when the double reciprical plot data was expressed as a Hill plot the increasing value of n_h (the slope) with increasing CoASH concentration can be rationalised in terms of the growth of the $1/[A]^2$ term. In this case a value of n_h greater than unity did not necessarily imply any co-operative interaction between the methanotrophic beta-ketothiolase and CoASH; moreover, the non-linearity of the reciprical plot in the presence of CoASH can be attributed to the reaction mechanism of this enzyme. Similar conclusions were drawn from detailed kinetic studies on beta-ketothiolase from mammalian tissue (Middleton, 1974; Huth et al., 1975).

The probable effect of CoASH addition to the system would, therefore, be to decrease the steady state level of the central acetyl-enzyme complex by converting it back to the enzyme-acetyl-CoA (E.AcCoA) derivative. The competitive inhibition of beta-ketothiolase by CoASH (with respect to acetyl-CoA) essentially reflects the distribution of the central, acetyl-enzyme complex between the enzyme-acetyl-CoA derivative (E.AcCoA) and the enzyme-acetoacetyl-CoA (E.AC.AcCoA) derivative (equation 5.6). Another point worth noting is that the competitive interaction of CoASH with respect to acetyl-CoA concentration is another means of diagnosing a ping-pong reaction mechanism (Cleland, 1970).

In the direction of acetoacetyl-CoA cleavage the kinetic properties of beta-ketothiolase were in accord with a ping-pong mechanism of action. This was apparent from the double reciprical plots, which exhibited parallel lines when acetoacetyl-CoA was varied



...(5.6)

Abbreviations: E, beta-ketothiolase; E-AC, the acetyl-enzyme covalent intermediate; AcCoA, acetyl-CoA; AcacCoA, acetoacetyl-CoA.

In the presence of CoASH the Lineweaver-Burk plot became parabolic, suggesting that the formation of the central (acetyl-enzyme) complex, from acetyl-CoA, can be reversed under these circumstances. This may be explained as follows: If one examines the general rate equation for an ordered two substrate reaction (equation 5.7) we find that it differs from equation 5.5, in that it contains a binary term $K_{aa}/[A]^2$.

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_{a1}}{[A]} + \frac{K_{a2}}{[A]} + \frac{K_{aa}}{[A]^2}\right] \qquad \ldots(5.7)$$

In this instance, the binary term accounts for the introduction of a reversable connection between the central enzyme substrate complex (E.AC) and the enzyme-acetyl-CoA (E.AcCoA) complex. This term becomes negligible only when substrate binding to enzyme (in the absence of products) can be regarded as irreversible (Cleland, 1970). Therefore, we have a term in $1/[A]^2$ which, if it is of significance will cause the plot of 1/v versus 1/[acetyl-CoA] to become parabolic instead of linear. The significance of this information is that it highlights the cause of the non-linearity of the condensation reaction in the

derived in this study, represents the sum of the two true K_m values of the enzyme for this substrate.

In order to satisfy the form of the rate equation represented in equation (5.5), the Lineweaver-Burk plot of the rate of acetoacetyl-CoA formation versus acetyl-CoA concentration (in the absence of products) should be linear. This criteria was satisfied by the methanotrophic enzyme since linearity was observed over a 8-fold range of substrate concentration. If the reaction was sequential, the reciprical plot would have been parabolic owing to the reversable steps between substrate addition and product release (Cleland, 1970).

One of the distinguishing features of a ping-pong reaction mechanism, as opposed to a sequential mechanism, is the formation of a stable central enzyme complex (Cleland, 1970). In view of the proposed mechanism of action of beta-ketothiolase (Gehring et al., 1968) (equation 5.6), involving the participation of a stable acetyl-enzyme complex (E.AC), the apparent irreversability of acetyl-CoA binding to the methanotrophic enzyme might possibly be due to the formation of the same derivative. Certainly, kinetic studies on this enzyme from other bacterial sources (Oeding & Schlegel, 1973; Berndt & Schlegel, 1975; Nishimura et al., 1978) support this reasoning.

5.4.1 KINETIC PROPERTIES OF BETA-KETOTHIOLASE

The kinetic behaviour of beta-ketothiolase from M. trichosporium OB3b was consistent with a ping-pong reaction mechanism as described by Cleland (1967, 1970). In this mechanism the substrates, acetyl-CoA and CoASH are never on the enzyme surface together. The substrate binding steps are separated by product release steps, which are considered irreversible under initial-rate conditions since product is at zero concentration. The rate equation which describes this reaction mechanism is indicated below (equation 5.4):

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_a}{[A]} + \frac{K_b}{[B]}\right] \qquad \ldots(5.4)$$

In the case of beta-ketothiolase, [A] and [B] represent identical molecules (acetyl-CoA), therefore, equation 5.4 can be rewritten as follows (equation 5.5):

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_{a1}}{[A]} + \frac{K_{a2}}{[A]}\right] \qquad \ldots(5.5)$$

(where K_{a1} is the K_m value for the first molecule of acetyl-CoA to bind to the enzyme and K_{a2} refers to the second molecule that binds). Although the enzyme will possess two K_m values for acetyl-CoA, it was not possible to derive these separately by steady state kinetics. Consequently, the K_m of beta-ketothiolase for acetyl-CoA (1.56 mM)

Bacterium	Zoogloea[1] ramigera	Azotobacter[2] beijerinkii	Clostridium[3] pasteurianum	Alcaligenes[4] eutrophus	Saccharomyces[5] cerevisiae	Methylosinus[6] trichosporium OB3b
Molecular Weight (including isoenzymes)	190,000	-	158,000 158,000)	147,000	140,000 (65,000)	145,000
MICHAELIS CONSTANTS						
Acetyl-CoA ($M \times 10^{-3}$)	0.39	0.9	2.5	0.39	-	1.56
Acetoacetyl-CoA ($M \times 10^{-6}$)	10	-	105-133	-	20 (350)	30
CoASH ($M \times 10^{-6}$)	8.5	-	18.9-28.8	-	160 (<1)	25
INHIBITORS						
Condensation reaction	CoASH	CoASH	CoASH	CoASH	-	CoASH
Cleavage reaction	NADH, NADPH	Acetoacetyl-CoA	Acetoacetyl-CoA, CoASH, NAD, Oxoglutarate	Acetoacetyl-CoA	CoASH, Acetoacetyl-CoA	CoASH

Table 5.3 Comparison of the Properties of Beta-ketothiolase from Various Microorganisms

1 Nishimura et al. (1978); 2 Senior & Dawes, (1973); 3 Berndt & Schlegel, (1975);
4 Oeding & Schlegel, (1973); 5 Kornblatt & Rudney, (1971a); 6 This thesis.

F3G-A and Procion Red H-3B, suggested that, in the former case, interaction between the enzyme and the dye was primarily electrostatic and probably a result of the anionic nature of this dye (Lascu et al., 1984). Conversely, Red H-3B, in part, fulfills the criteria of an affinity ligand for beta-ketothiolase in that the enzyme was effectively eluted from this dye-ligand by its cofactor, CoASH. The difference in elution procedures which were possible following the binding of beta-ketothiolase to either Blue F3G-A or Red H-3B was subsequently reflected in the degree of purification achieved from each of these dye-ligands.

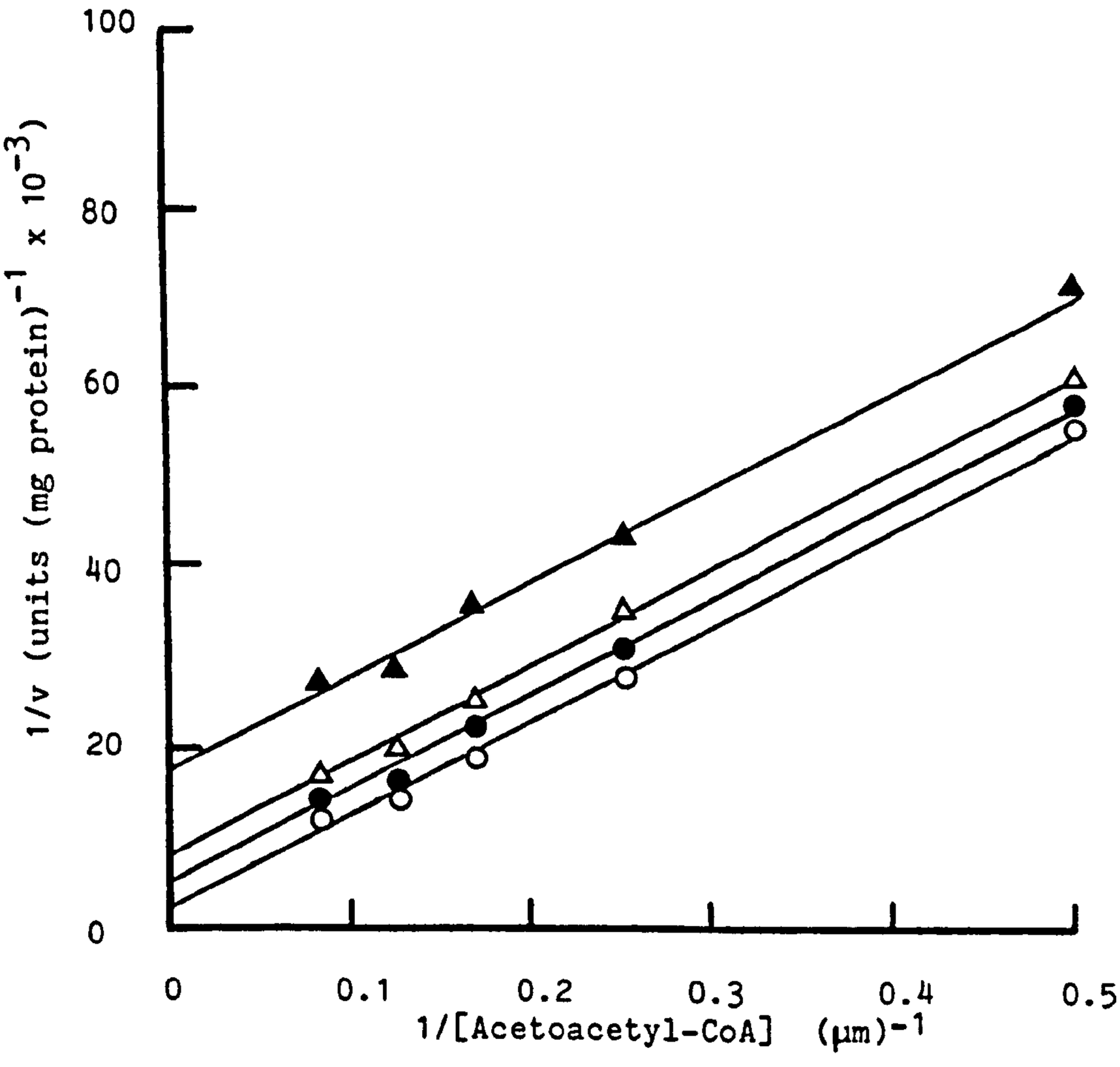
From the evidence obtained in this study it appears that beta-ketothiolase from M. trichosporium OB3b is present as a single isoenzyme and not as multiforms as in the case of S. cerevisiae (Kornblatt & Rudney, 1971a) or C. pasteurianum (Berndt & Schlegel, 1975). Molecular weight studies on the methanotrophic enzyme suggest that the enzyme is composed of four identical subunits (38,000 daltons) giving a native molecular weight of 145,000. This value was significantly higher than the pH 7.8 isoenzyme from S. cerevisiae (M.W. 65,000, Kornblatt & Rudney, 1971a) and lower than the enzyme from Z. ramigera I-16-M which has a molecular weight of 190,000 (Nishimura et al., 1978). It is, however, in the same order of magnitude as the molecular weight of the pH 5.3 thiolase from S. cerevisiae (M.W. 140,000) as well as the thiolase from A. eutrophus H16 (M.W. 147,000 - 150,000). A summary of a range of properties of beta-ketothiolases, from various sources is provided in Table 5.3.

5.4 DISCUSSION

The scheme developed in this study for the purification of beta-ketothiolase from M. trichosporium OB3b includes the first description of the use of triazine dye affinity chromatography in the purification of this enzyme from any source. This technique, in combination with the discovery of CoASH as a specific elutant of beta-ketothiolase from Red H-3B Sepharose, facilitated the production of a high yield of near homogeneous enzyme in two steps. By way of comparison, methods used previously to purify beta-ketothiolase from both bacterial and mammalian sources have relied specifically on conventional chromatographic techniques and are characterised by multi-step procedures resulting typically in low yields of enzyme (Oeding & Schlegel, 1973; Huth et al., 1975; Nishimura et al., 1978).

Although triazine dye affinity chromatography has been used with success in a large number of enzyme and protein purifications, there has been little optimisation of binding and elution protocols for requisite proteins (Scopes, 1986). The significance in evaluating these variables was adequately demonstrated in this study, where both the choice of dye ligand and the method of enzyme elution were critical to the success of the purification scheme. In the first instance the choice of dye ligand appears to be important in determining selectivity for beta-ketothiolase and also its mode of interaction with the enzyme. A detailed examination on the binding of beta-ketothiolase to the closely related dye-ligands, Cibacron Blue

(1)


100
80
60
40
20
0
1/v (units (mg protein)$^{-1}$ x 10^{-3})
0
0.1
0.2
0.3
0.4
0.5
1/[Acetoacetyl-CoA] $(\mu m)^{-1}$

(2)

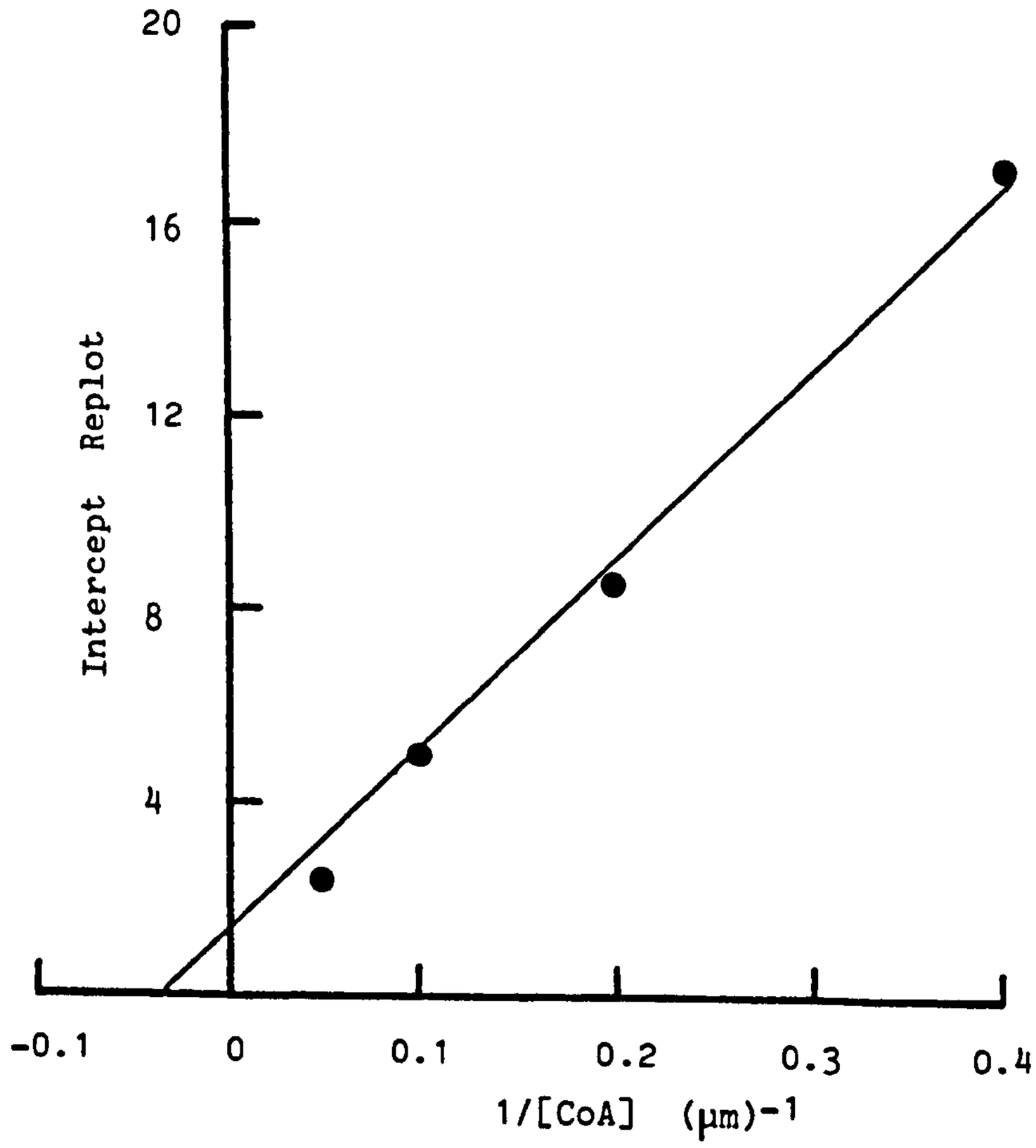

20
16
12
8
4
Intercept Replot
-0.1
0
0.1
0.2
0.3
0.4
1/[CoA] $(\mu m)^{-1}$

Figure 5.11 Dependence of the Activity of Beta-Ketothiolase on the Concentration of Acetoacetyl-CoA at Different Levels of CoASH

Cuvettes contained in a total reaction volume of 1 ml: Tris-HCl buffer (100 mM, pH 8.35); $MgCl_2$ (20 mM); acetoacetyl-CoA (2.5 - 20 μM) and purified enzyme preparation (10.7 μg of protein). Concentration of CoASH (μM): (▲) 2.5; (△) 5; (●) 10; (○) 20.

(1) Lineweaver-Burk plot.

(2) Secondary re-plot of data obtained from (1).

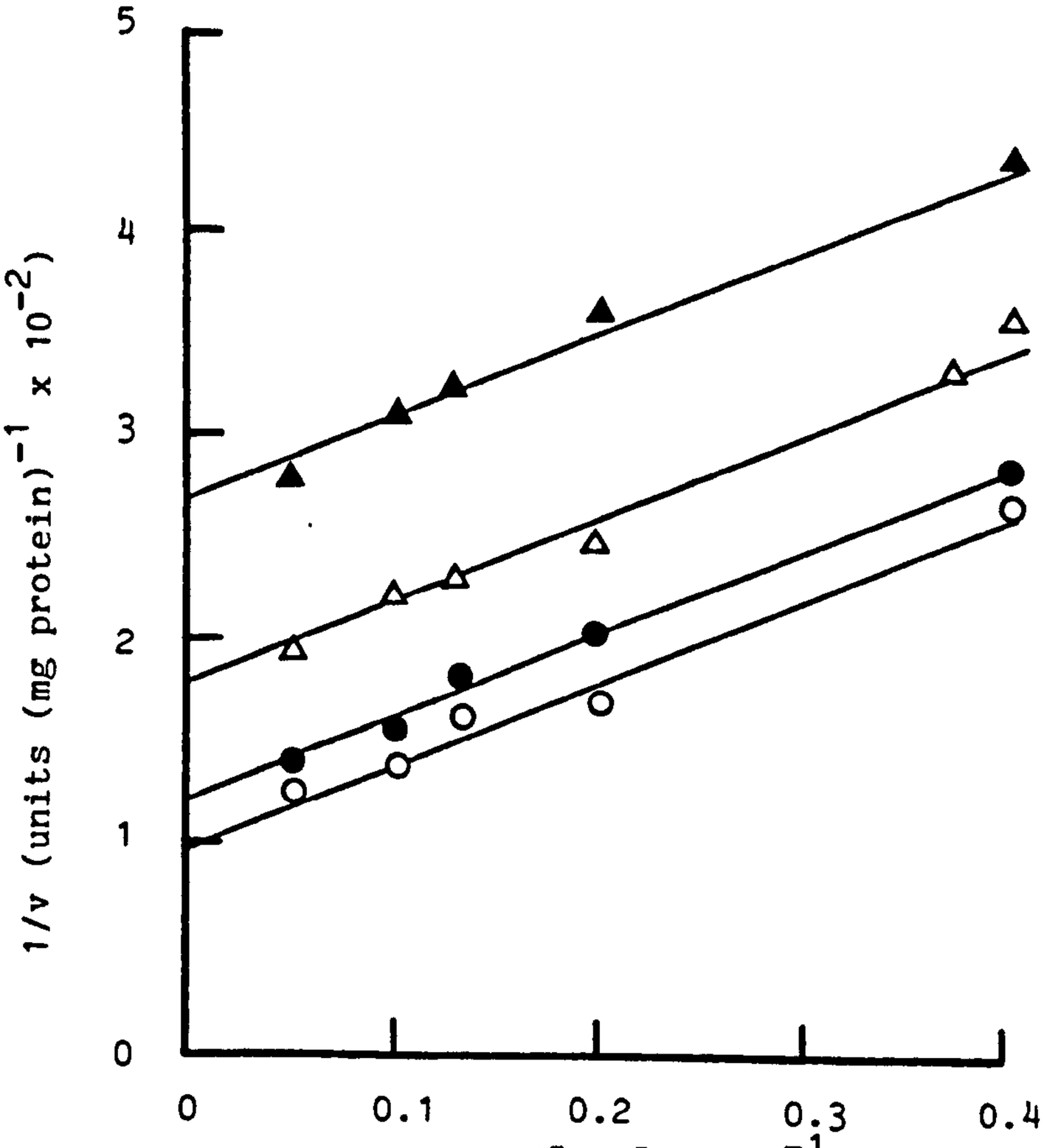
From the intercept replot of Figure 5.10.2 the apparent K_m for acetoacetyl-CoA was estimated to be 30 x 10^{-6} M (Figure 5.10.3).

Acetoacetyl-CoA saturation curves were measured at four fixed levels of CoASH (2.5, 5, 10 and 20 μM). The enzyme appeared to exhibit normal Michaelis-Menten kinetics and the Lineweaver-Burk plot of this data (Figure 5.11.1) gave a series of parallel lines, indicative of a ping-pong reaction mechanism. From the intercept replot of Figure 5.11.1 the apparent K_m for CoASH was estimated to be 25 x 10^{-6} M (Figure 5.11.2).

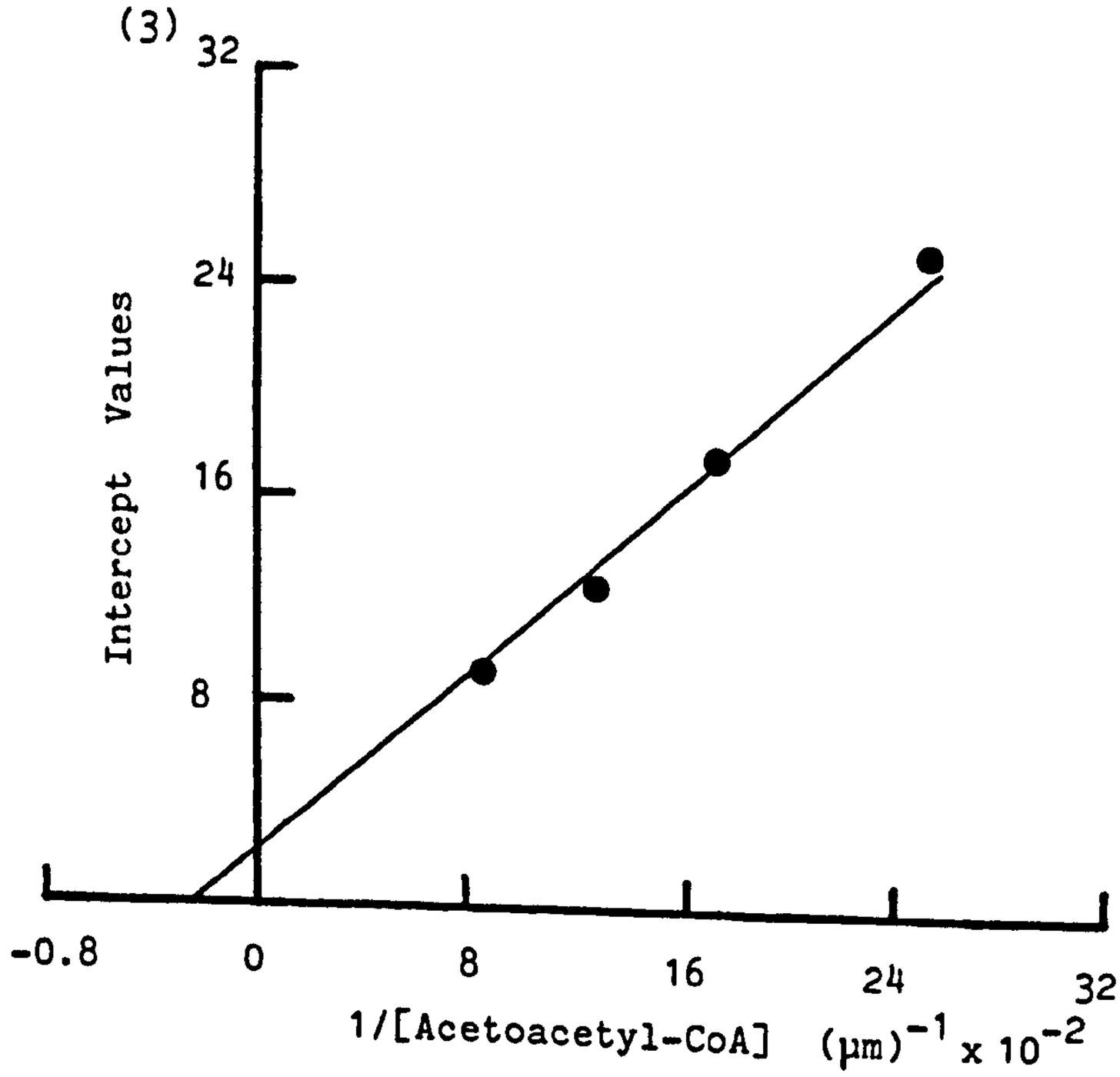
5.3.7 INFLUENCE OF METABOLITES ON BETA-KETOTHIOLASE ACTIVITY

The results obtained from investigating the cleavage and condensation reactions coincided so far as beta-ketothiolase was recognised as a regulatory enzyme. The question arose as to whether the activity of the enzyme was influenced by metabolites other than substrate and reaction products. Therefore, this study examined a wide range of metabolites that would possibly function as effectors of either the condensation or cleavage reaction; these have been described previously in Section 3.2.1. During this study no significant inhibition of beta-ketothiolase was observed in the presence of the metabolites tested and thus one can conclude that this enzyme is regulated through the relative levels of its reaction substrates and or products.

(2)


5
4
3
2
1
0
1/v (units (mg protein)$^{-1}$ x 10^{-2})
0
0.1
0.2
0.3
0.4
1/[CoA] (μM)$^{-1}$

(3)


32
24
16
8
Intercept Values
-0.8
0
8
16
24
32
1/[Acetoacetyl-CoA] (μm)$^{-1}$ x 10^{-2}

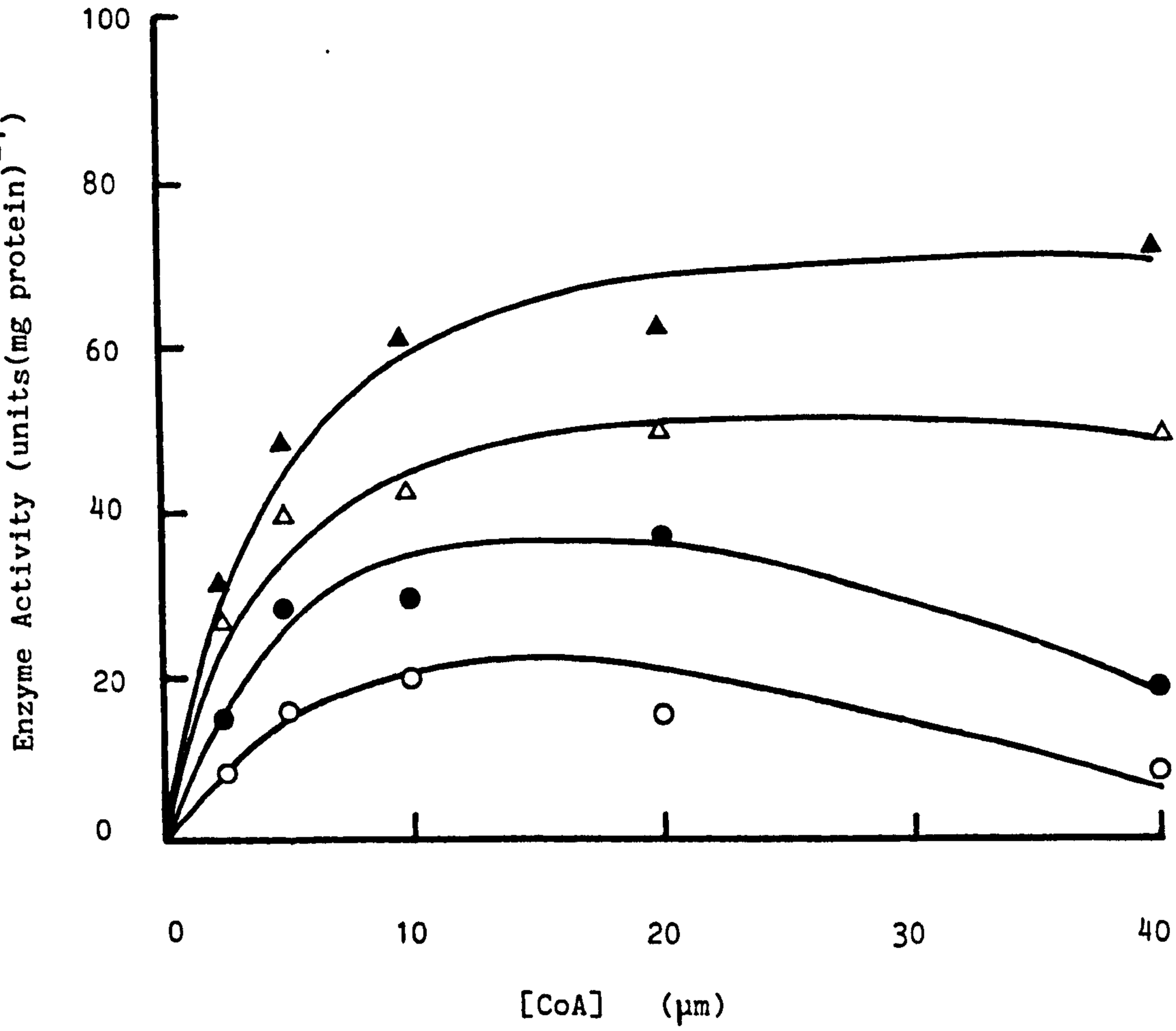

Enzyme Activity (units(mg protein)$^{-1}$)
100
80
60
40
20
0
0
10
20
30
40
[CoA] (μm)

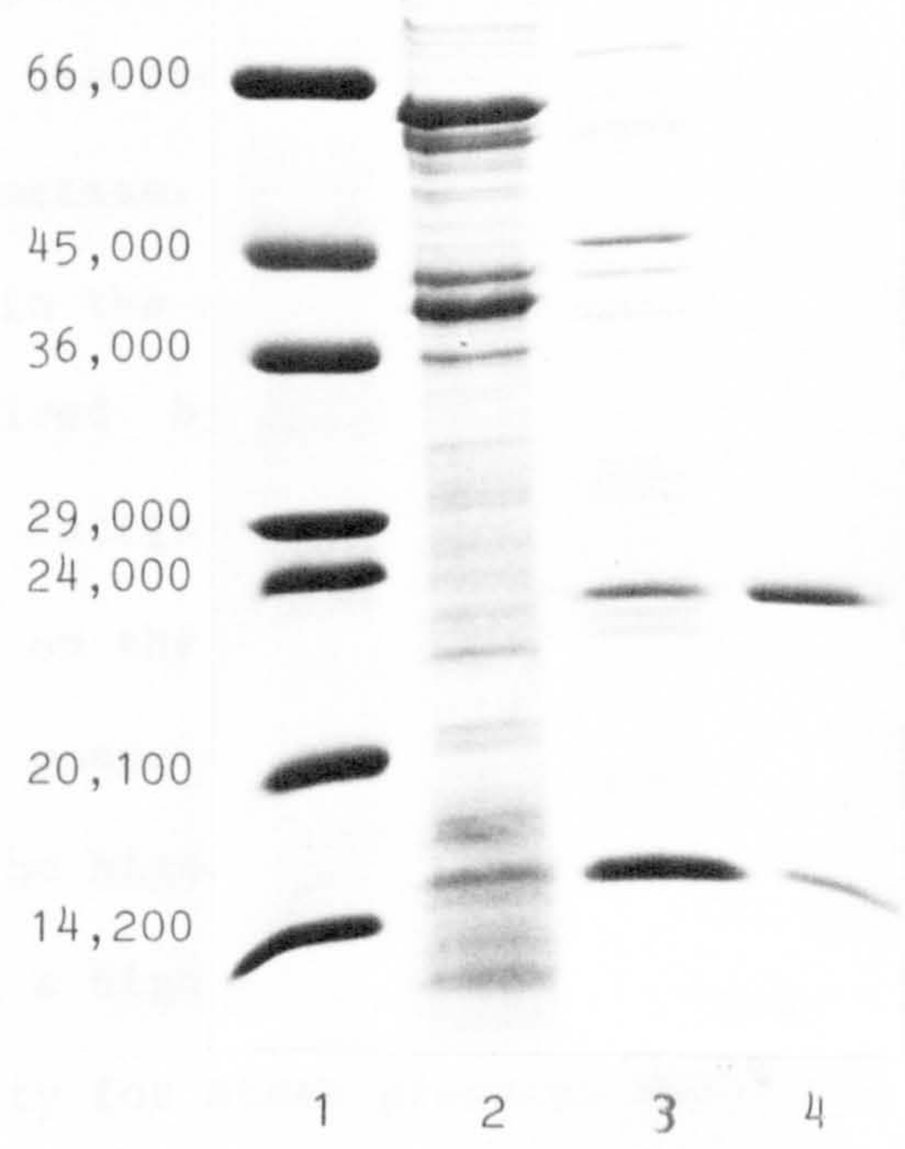
Figure 5.10 Dependence of the Activity of Beta-Ketothiolase on the Concentration of CoASH at Different Levels of Acetoacetyl-CoA

Cuvettes contained in a total reaction volume of 1 ml: Tris-HCl buffer (100 mM, pH 8.35); $MgCl_2$ (20 mM); CoASH (2.5 - 40 uM) and purified enzyme preparation (10.7 µg of protein). Concentration of acetoacetyl-CoA (µM): (○) 4; (●) 6; (△) 8; (▲) 12.

(1) Substrate (CoASH) saturation curves.

(2) Lineweaver-Burk plot of data shown in (1).

(3) Secondary re-plot of intercept data obtained from (2).


66,000
45,000
36,000
29,000
24,000
20,100
14,200
1
2
3
4
Acetoacetyl-CoA
reductase

Red H-8B, Blue F3G-A, Blue MX-R, Blue MX-2R, Orange MX-2R and Green H-E4BD.

Small scale studies (5 ml column) revealed that each dye-conjugate bound acetoacetyl-CoA reductase from the crude extract of this organism. Although the study was largely qualitative, early indications suggested that the enzyme possessed a high affinity for these ligands. This was highlighted by the observation that although both beta-ketothiolase and 3-HBD were bound by each conjugate, they were competitively desorbed prior to the saturation of each column by acetoacetyl-CoA reductase. In light of this information, the choice of dye ligand(s) used in the subsequent purification of acetoacetyl-CoA reductase was evolved by way of the strategy presented by Scopes (1986), in which he categorised a large number of triazine dyes into five groups, based on their binding affinity for a wide range of test proteins. Group one possessed the lowest capacity for protein loading and group five the highest. Consequently, by careful selection of a dye which possessed a high affinity for the enzyme of choice, coupled with a low affinity for other proteins one might expect to achieve a high degree of purification. By adopting this approach, it was possible to minimise the potentially time consuming evaluation of each dye ligand for its suitability in the purification of acetetoacetyl-CoA reductase from this organism. Of those dyes examined initially, Blue MX-R and Blue MX-2R were selected for further study. These dyes belonged to groups two and three respectively, the remaining dyes indicated previously belonged to either groups four or five.

Small scale binding studies of acetoacetyl-CoA reductase to Blue MX-2R and Blue MX-R were conducted under identical chromatographic conditions, with a dye substitution value of 4 µmol.dye/ml Sepharose in each case. As predicted by Scopes (1986), Blue MX-2R (16 +/- 1.1 units of acetoacetyl-CoA reductase/ml Sepharose gel) possessed a higher binding capacity for acetoacetyl-CoA reductase than Blue MX-R (10 +/- 0.8 units of acetoacetyl-CoA reductase/ml Sepharose gel). Although the purification achieved following salt elution of acetoacetyl-CoA reductase from Blue MX-R was consistently higher than that achieved following salt elution of the enzyme from Blue MX-2R, the latter was chosen to conduct the first step in the purification scheme on the basis of its higher binding capacity for the enzyme from the crude extract. As noted during the purification of 3-HBD from this organism (Section 3.3), the level of purification achieved was dependant upon the protein loading. By utilising the maximum binding capacity of Blue MX-2R for acetoacetyl-CoA reductase it was possible to routinely achieve an initial purification value in excess of 40-fold.

Specific cofactor desorption of acetoacetyl-CoA from Blue MX-R Sepharose was explored following its initial purification from Blue MX-2R Sepharose. In this respect, both NADP and NADPH were effective in eluting the enzyme from this dye-conjugate, which was not altogether surprising in view of the nature of these dyes and the cofactor dependancy of this enzyme. The study, nevertheless, revealed that NADPH was demonstrably superior in eluting the enzyme from MX-R Sepharose by virtue of the lower concentration required to initiate the process. Neither NAD, NADH, ATP or CoASH were effective in eluting

the enzyme from Blue MX-R, when tested over a range of concentrations (0 to 5 mM), thus highlighting the specificity of the interaction between the dye and the cofactor dependant enzyme.

6.3.2 STABILITY OF ACETOACETYL-COA REDUCTASE

Acetoacetyl-CoA reductase was stable in the crude extract following storage at $4^{o}C$ for 72 h. However, the addition of a sulphydryl reagent was necessary in order to maintain enzyme activity following its initial purification from Blue MX-2R. Typically, a loss in enzyme activity in excess of 30 % was noted following storage at $4^{o}C$ for 24 h in the absence of a sulphydryl reagent. The general conclusion drawn from this information was that the enzyme possibly possessed a thiol group essential to catalytic activity. However, this was not investigated further in this study.

6.3.3 MOLECULAR WEIGHT OF ACETOACETYL-COA REDUCTASE

The molecular weight of acetoacetyl-CoA reductase was determined following its elution from a column of Sephacryl S-300, standardised with known molecular weight proteins. By this technique a molecular weight of 46,000 daltons was estimated for the native protein. (Figure 6.2). A subunit molecular weight of 23,000 daltons was determined by comparing its mobility (Rf) on a 12 % SDS polyacrylamide gel with those of standard molecular weight proteins (Figure 6.3). The data suggests that acetoacetyl-CoA reductase from this organism was probably dimeric and composed of two identical subunits.

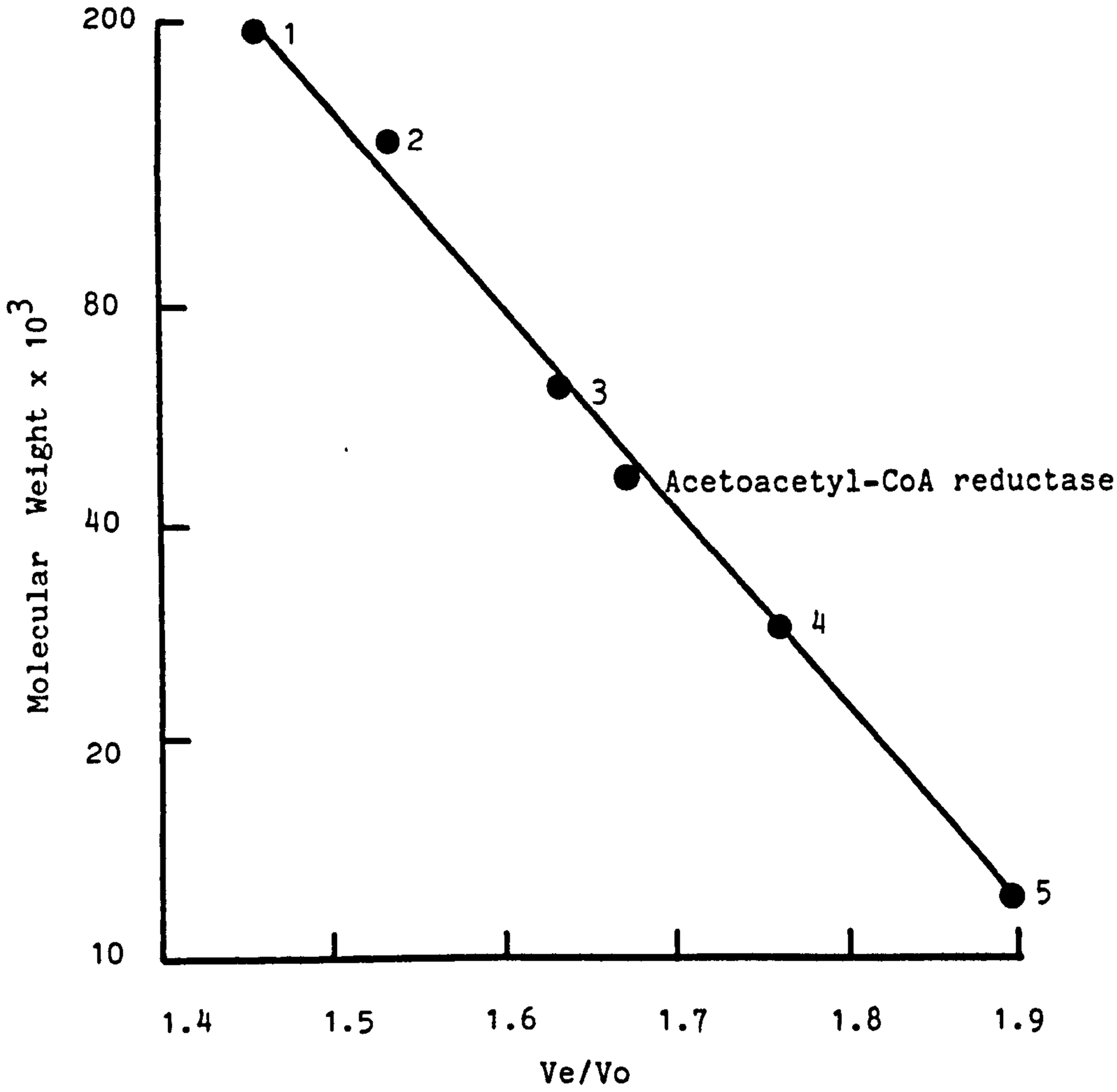


Figure 6.2 Molecular Weight Determination of Acetoacetyl-CoA Reductase from M. trichosporium OB3b by Sephacryl S-300 Gel Filtration

The elution volume of acetoacetyl-CoA reductase from a column of Sephacryl S-300 was compared to proteins of known molecular weight. 1. Beta-Amylase; 2. Alcohol Dehydrogenase; 3. Albumin, Bovine; 4. Carbonic Anhydrase; 5. Cytochrome C. (see section 2.9 for further details).

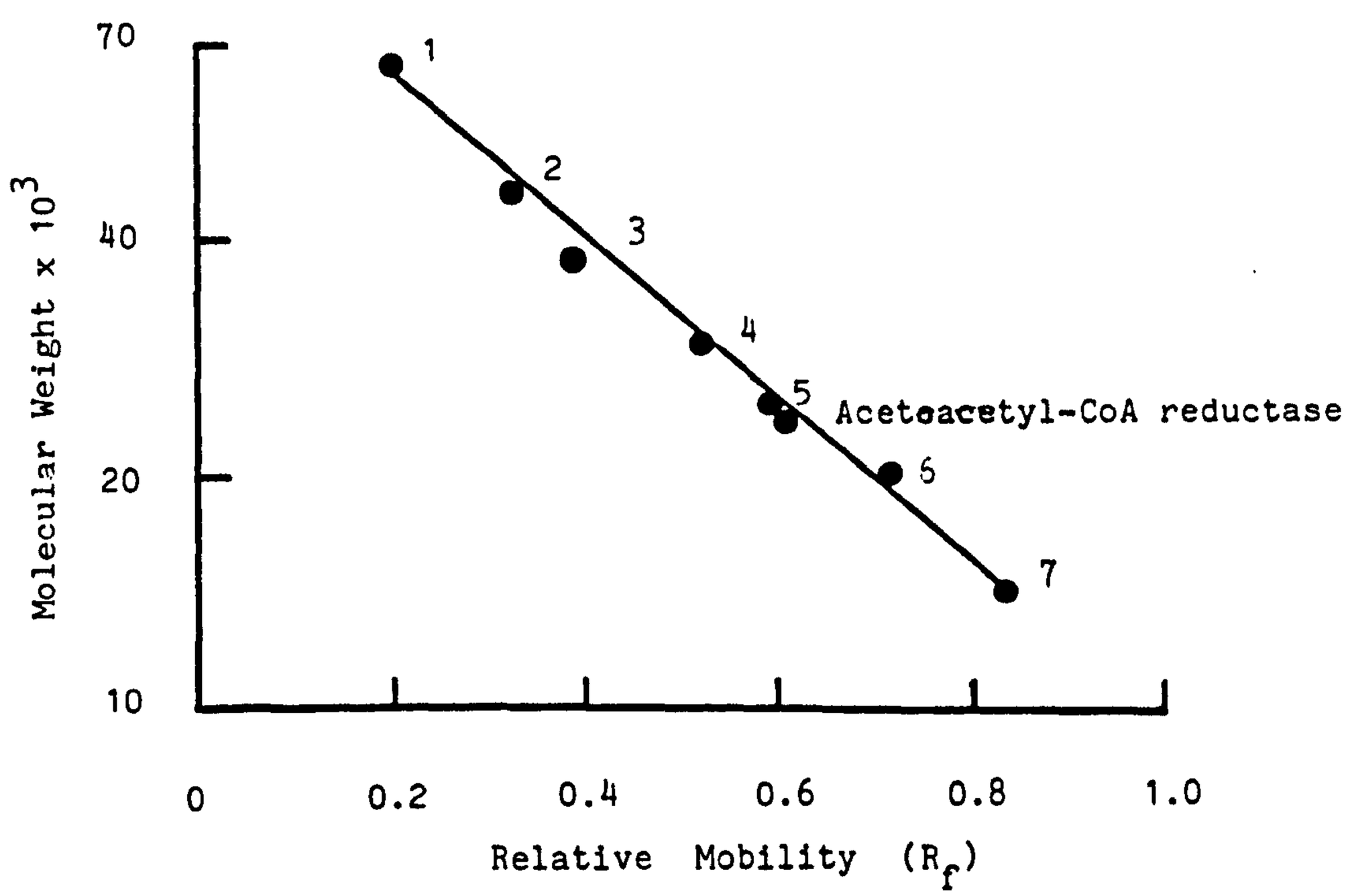


Figure 6.3 Determination of the Subunit Molecular Weight of Acetoacetyl-CoA Reductase from M. trichosporium OB3b by SDS Polyacrylamide Gel Electrophoresis

The relative mobility of the dissociated enzyme on a 12 % polyacrylamide gel containing SDS, was compared to proteins of known molecular weight. 1. Albumin, Bovine; 2. Albumin, Egg; 3. Glyceraldehyde-3-phosphate dehydrogenase; 4. Carbonic Anhydrase; 5. Trypsinogen; 6. Trypsin Inhibitor, Soybean; 7. Alpha-Lactalbumin. (see section 2.10 for further details).

6.3.4 CATALYTIC PROPERTIES OF ACETOACETYL-COA REDUCTASE

During this study two different spectrophotometric assays were used to measure the activity of acetoacetyl-CoA reductase; these have been described previously (section 6.2). Routine measurement of acetoacetyl-CoA reductase activity was achieved by monitoring the reoxidation of NADPH at 340 nm. It was not possible, however, to estimate the K_m for acetoacetyl-CoA and NADPH by this method owing to the high affinity of the enzyme for its substrate. Instead these constants were determined by the magnesium-enol disappearance assay. As a consequence of the higher molar extinction coefficient of acetoacetyl-CoA (E_m = 17 x 10^3 $1.mol^{-1}.cm^{-1}$ at pH 8.2) compared with NADPH (E_m = 6.22 x 10^3 $1.mol^{-1}.cm^{-1}$), the former assay is three times more sensitive than the latter measurement of NADPH reoxidation.

The pH profiles obtained for the measurement of acetoacetyl-CoA reductase activity by both assay methods are illustrated in Figure 6.4. Clearly, the pH profiles were dissimilar, as were the pH optimum obtained in each case (pH 8.8 by the NADPH reoxidation assay and pH 8.2 by the magnesium-enolate assay). The reason(s) for such marked differences between the two assays was not altogether clear. One possible explanation might be that the NADPH reoxidation assay was a true estimation of the effect of pH on enzyme activity, whereas the magnesium-enol method was not a true estimation of this value. Since pH and magnesium ions dictate the equilibrium between the keto (substrate) and enol forms of acetoacetyl-CoA (see section 5.3.5), a secondary effect on the reaction rate, in addition to the effect of pH on enzyme activity might exist in this case. It was probably worth

Figure 6.4 Effect of pH on the Activity of Acetoacetyl-CoA Reductase from M. trichosporium OB3b

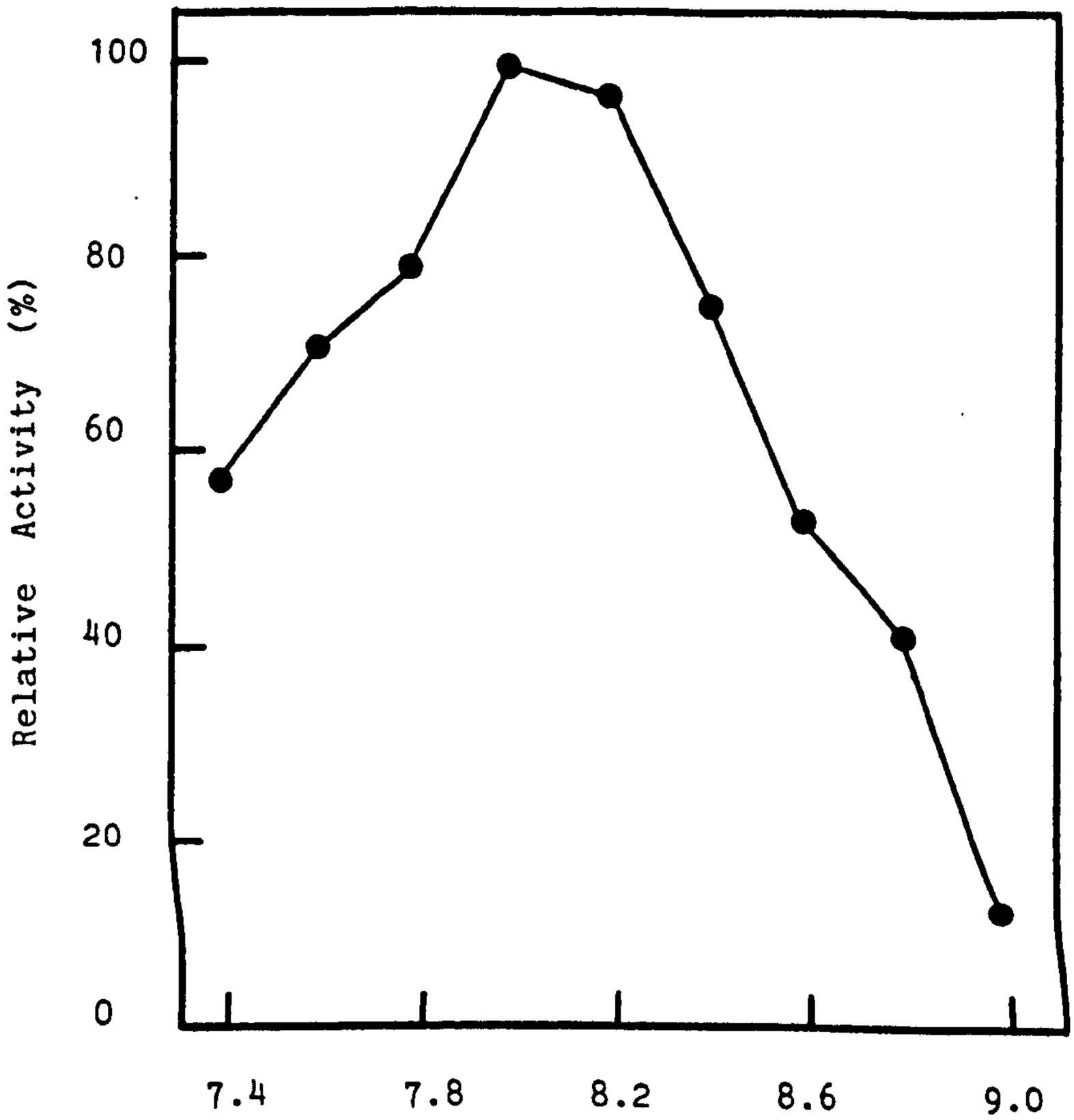
The reduction of acetoacetyl-CoA by acetoacetyl-CoA reductase was measured at the appropriate pH by one of the two methods described in section 6.2.2. The relative activities obtained in (1) represent a correction for the pH dependant change in the molar extinction coefficient of acetoacetyl-CoA as described in section 5.3. Buffers (100 mM) used in this study were as follows:

(○) Phosphate buffer; (●) Tris-HCl buffer.

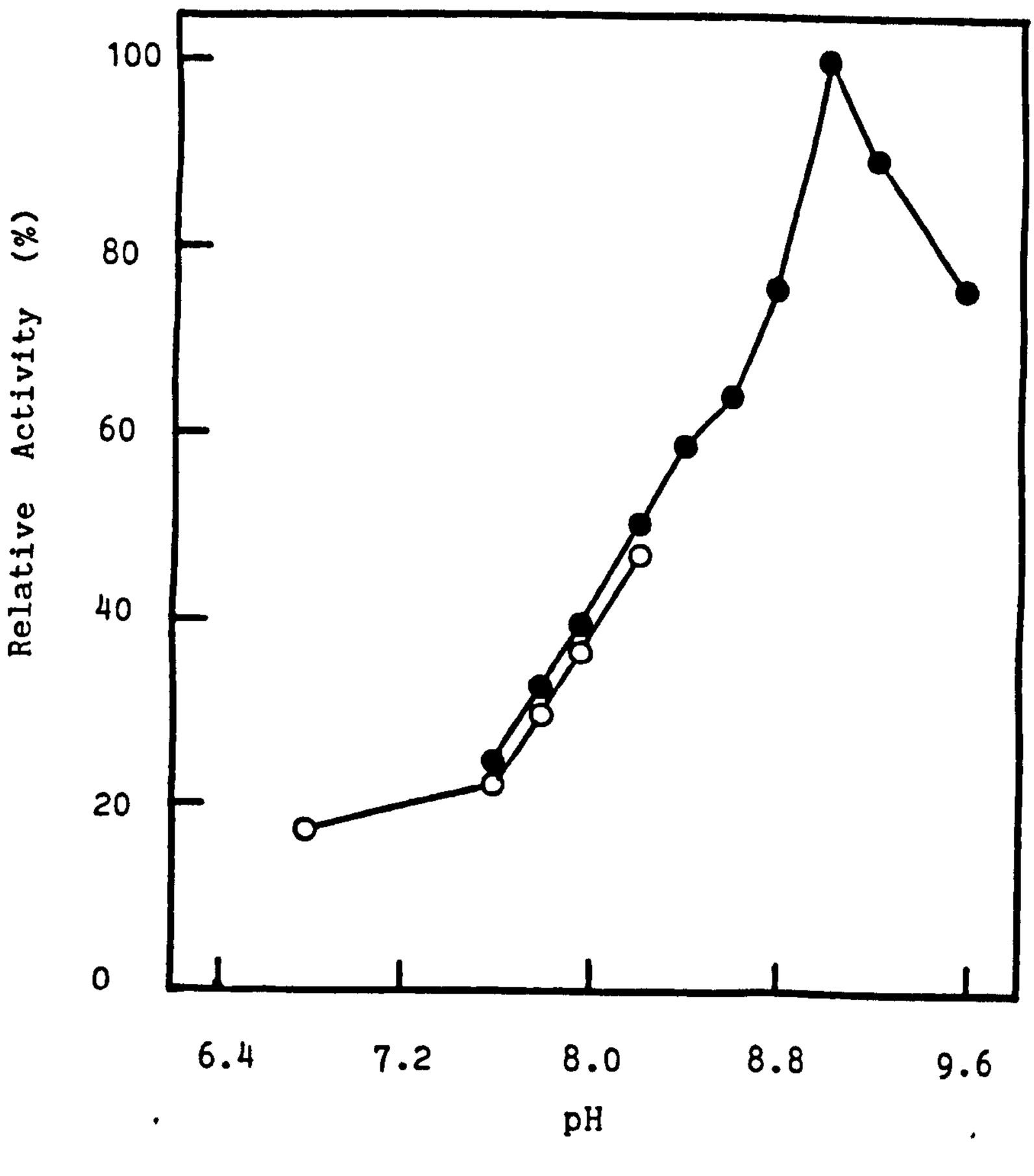
(1) Magnesium-enolate method (303 nm).

(2) NADPH oxidation (340 nm).

(1)


100
80
60
40
20
0
Relative Activity (%)
7.4
7.8
8.2
8.6
9.0
pH

(2)


100
80
60
40
20
0
Relative Activity (%)
6.4
7.2
8.0
8.8
9.6
pH

noting, however, that the maximum specific activity obtained by each assay method was similar.

The apparent K_m of acetoacetyl-CoA reductase for both acetoacetyl-CoA (Figure 6.5) and NADPH (Figure 6.6) was determined by the method of Lineweaver-Burk (1934) using the magnesium-enolate assay. These were estimated to be 4.5×10^{-6} M and 42×10^{-6} M respectively. The V_{max} for the reduction of acetoacetyl-CoA by this enzyme was subsequently calculated to be 40 units $(mg\ protein)^{-1}$. Furthermore, the enzyme was highly specific for its cofactor, NADPH and could not be replaced by NADH.

The possibility that M. trichosporium OB3b may operate an alternative pathway for the production of D(-)-3-hydroxybutyryl-CoA, similar to that described in R rubrum (Moskowitz & Merrick, 1969) was also explored in this study. However, all attempts to detect crotonase activity in this organism was unsuccesful, which led to the conclusion that D(-)-3-hydroxybutyryl-CoA formation prior to its polymerisation to form PHB was probably channelled through acetoacetyl-CoA reductase.

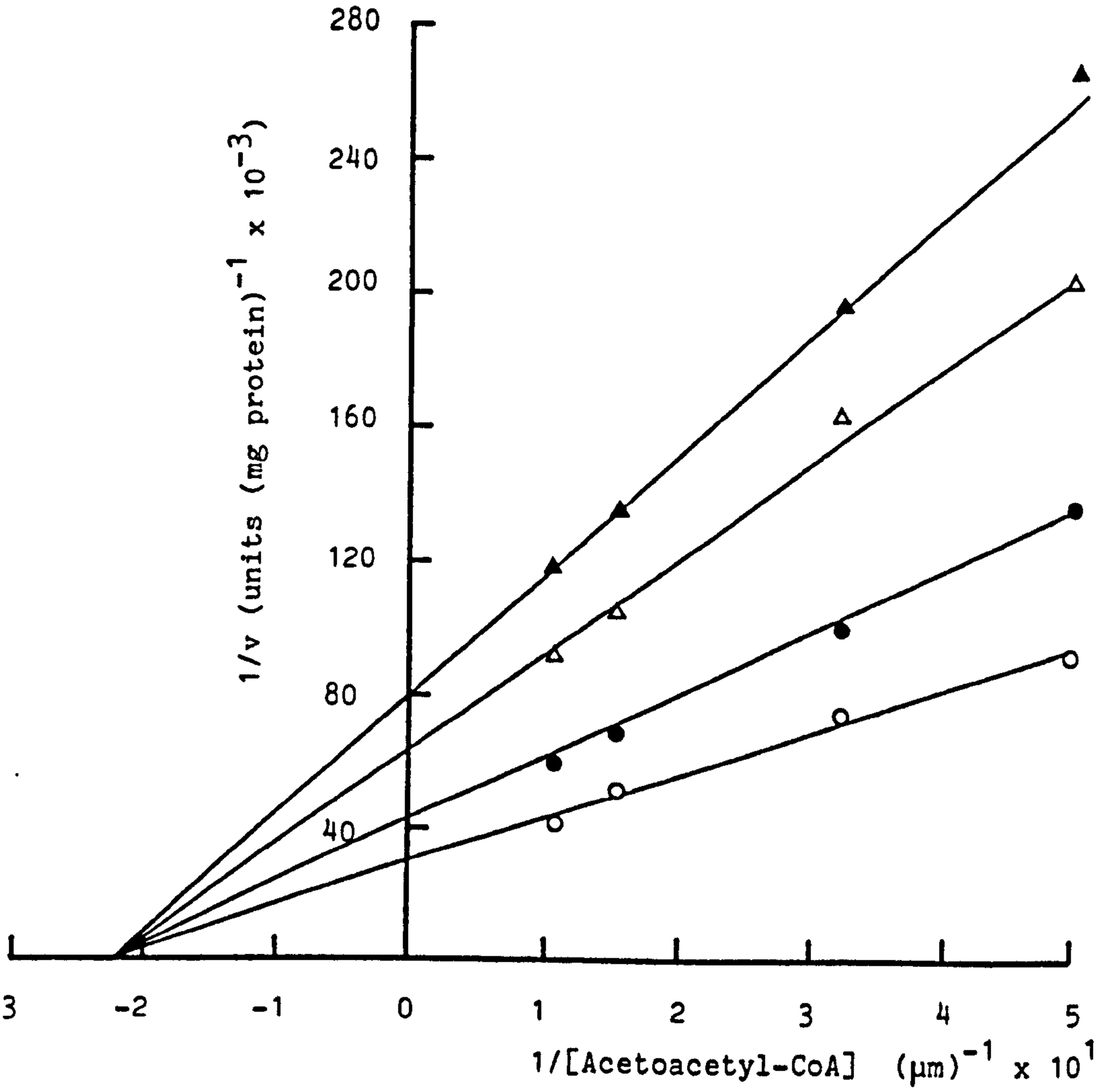


Figure 6.5 Lineweaver-Burk Transformation of Kinetic Data for the Reduction of Acetoacetyl-CoA, Catalysed by Acetoacetyl-CoA Reductase at Different Initial Levels of Acetoacetyl-CoA.

Cuvettes contained in a total reaction volume of 1 ml (final concentration): Tris-HCl buffer (100 mM, pH 8.2); $MgCl_2$ (20 mM); acetoacetyl-CoA (2.0 - 9.0 μM); purified enzyme preparation (0.29 μg of protein). Concentration of NADPH (mM): (○) 0.15; (●) 0.05; (△) 0.025; (▲) 0.015.

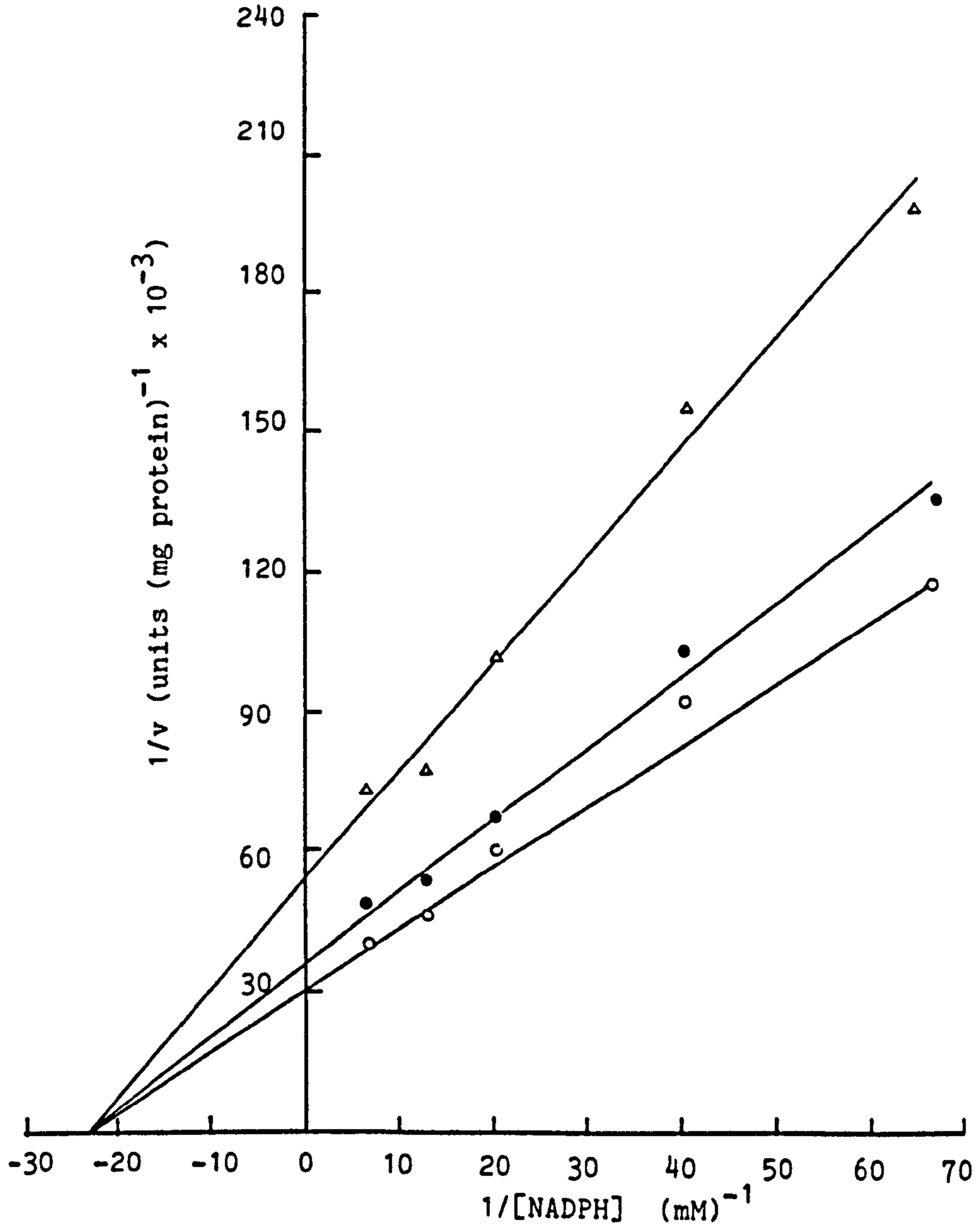


Figure 6.6 Lineweaver-Burk Transformation of Kinetic Data for the Reduction of Acetoacetyl-CoA, Catalysed by Acetoacetyl-CoA Reductase, at Different Initial Levels of NADPH

Cuvettes contained in a total reaction volume of 1 ml (final concentration): Tris-HCl buffer (100 mM, pH 8.2); $MgCl_2$ (20 mM); NADPH (0.015 - 0.15 mM); purified enzyme preparation (0.29 µg of protein). Concentration of acetoacetyl-CoA (µM): (○) 9.0; (●) 6.0; (△) 3.0.

6.3.5 THE REGULATION OF ACETOACETYL-COA REDUCTASE

The kinetic analysis of beta-ketothiolase from M. trichosporium OB3b (section 5.3) highlighted its importance in the regulation of PHB synthesis (acetoacetyl-CoA formation). The study also concluded that the regulation of acetoacetyl-CoA metabolism was important in the control of PHB synthesis, in view of its effect on the equilibrium of the reaction catalysed by beta-ketothiolase. A study on the influence of a number of metabolites on acetoacetyl-CoA reductase activity was therefore assessed, the range and concentration of which have been described previously (section 3.3). Of those compounds tested, no inhibition of acetoacetyl-CoA reductase activity was noted, apart from the substrate and reaction products of the enzyme. Substrate (acetoacetyl-CoA) and product (NADP and D(-)-3-hydroxybutyryl-CoA) inhibition of the enzyme is considered in more detail in the next section.

6.3.5.1 THE KINETICS OF SUBSTRATE AND PRODUCT INHIBITION OF ACETOACETYL-COA REDUCTASE

In order to assess the relative potency of NADP and D(-)-3-hydroxybutyryl-CoA inhibition on acetoacetyl-CoA reductase from M. trichosporium OB3b a detailed kinetic study on the effect of each product inhibitor on the activity of the enzyme was initiated. The object of the study was primarily to determine the type and extent of inhibition exerted by each compound on the enzyme, by slope and intercept analysis of Lineweaver-Burk plots of the data. In this way it was envisaged that one might speculate on the significance of each

inhibitor in vivo and thus highlight the significance of acetoacetyl-CoA reductase in the regulation of PHB synthesis.

In the presence of saturating acetoacetyl-CoA and a variable NADPH concentration, NADP inhibition of acetoacetyl-CoA reductase was competitive with respect to NADPH; this was illustrated by the Lineweaver-Burk plot presented in Figure 6.7.1. Clearly, NADP affected the slopes but not the intercepts of the double reciprical plot. This information was consistent with a reaction mechanism in which both molecules can be bound by the same enzyme form. A secondary replot (Figure 6.7.2) of the slope values obtained from the original data revealed that NADP acted as a linear inhibitor of this enzyme with a K_{is} value of 0.12 mM.

D(-)-3-hydroxybutyryl-CoA inhibition of acetoacetyl-CoA reductase was studied under two sets of conditions. In the presence of saturating acetoacetyl-CoA and variable NADPH concentration, D(-)-3-hydroxybutyryl-CoA acted as a non-competitive inhibitor of the enzyme. This was characterised by the Lineweaver-Burk plot of the data (Figure 6.8.1), where both the slope and the intercept increased in the presence of this compound. Since both slope and intercept were affected by the inhibitor, the inhibitor constant K_i was obtained by secondary replots of both the slope (Figure 6.8.2) and intercept (Figure 6.8.3) values of the original data from Figure 6.8.1. By this method a K_{is} (1.1 mM) and K_{ii} (1.2 mM) were obtained. Similarly under conditions where NADPH was saturating and the acetoacetyl-CoA concentration was varied, D(-)-3-hydroxybutyryl-CoA was a non-competitive inhibitor of the enzyme (Figure 6.9.1). The K_{is}

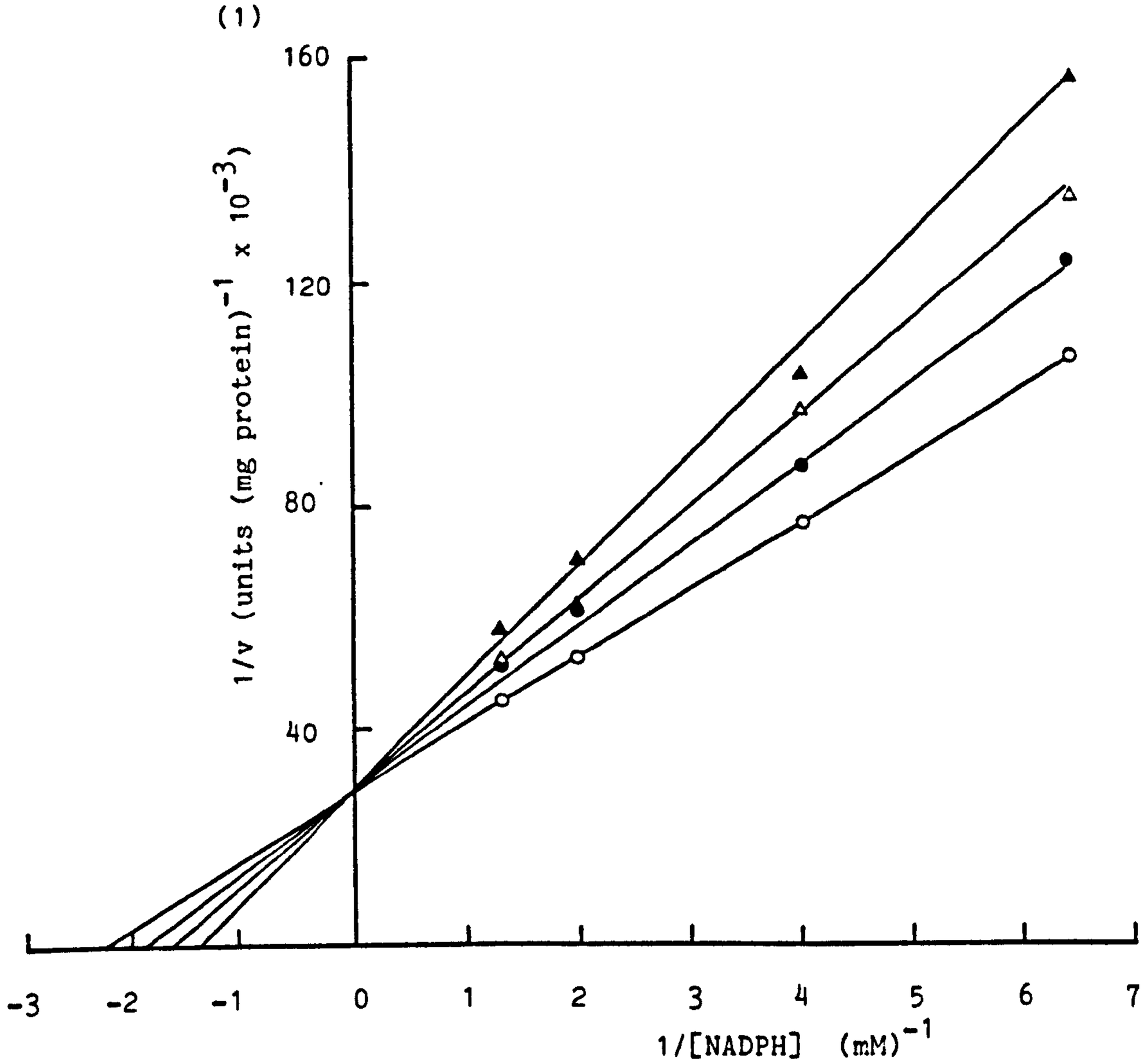
Figure 6.7 The Effect of NADP Concentration on the Initial Velocity of the Reaction Catalysed by Acetoacetyl-CoA Reductase, in the Presence of Fixed, Saturating Acetoacetyl-CoA and Variable NADPH Concentration

Cuvettes contained in a total reaction volume of 1 ml (final concentration): Tris-HCl buffer (100 mM, pH 8.2); $MgCl_2$ (20 mM); acetoacetyl-CoA (20 μM); NADPH (0.015 - 0.075 mM); purified enzyme preparation (0.29 μg of protein). Concentration of NADP (mM): (○) 0; (●) 0.025; (△) 0.05; (▲) 0.075.

(1) Lineweaver-Burk transformation of data.

(2) Secondary re-plot of slope values obtained from data shown in (1).

(1)


160
120
80
40
1/v (units (mg protein)$^{-1}$ x 10^{-3})
-3
-2
-1
0
1
2
3
4
5
6
7
1/[NADPH] (mM)$^{-1}$

(2)

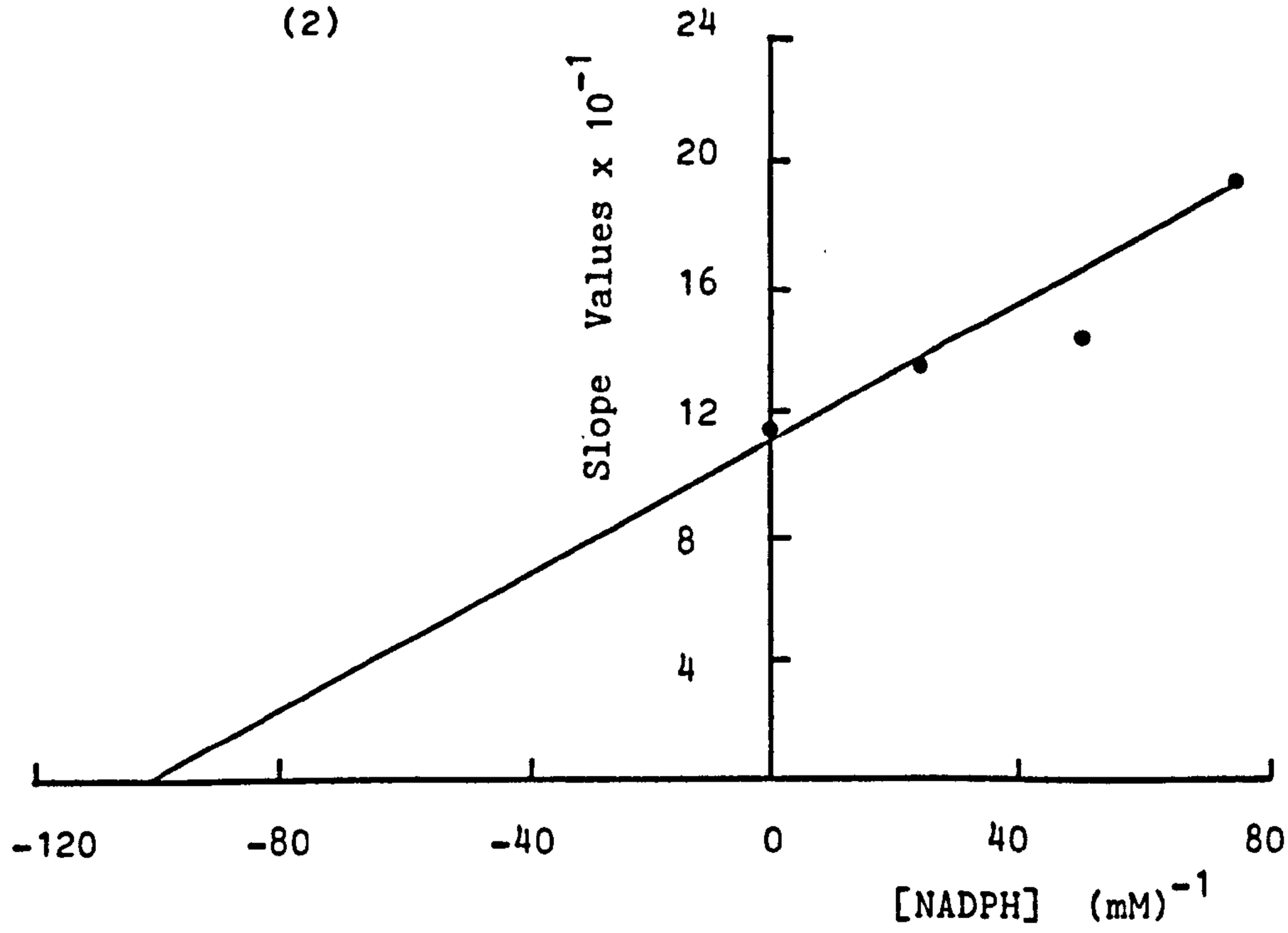

24
20
16
12
8
4
Slope Values x 10^{-1}
-120
-80
-40
0
40
80
[NADPH] (mM)$^{-1}$

Figure 6.8 The Effect of D(-)-3-Hydroxybutyryl-CoA Concentration on the Initial Velocity of the Reaction Catalysed by Acetoacetyl-CoA Reductase, in the Presence of Fixed, Saturating Acetoacetyl-CoA and Variable NADPH Concentration

Cuvettes contained in a total reaction volume of 1 ml (final concentration): Tris-HCl buffer (100 mM, pH 8.2); $MgCl_2$ (20 mM); acetoacetyl-CoA (20 μM); NADPH (0.015 - 0.15 mM); purified enzyme preparation (0.29 μg of protein).

Concentration of D(-)-3-hydroxybutyryl-CoA (mM):

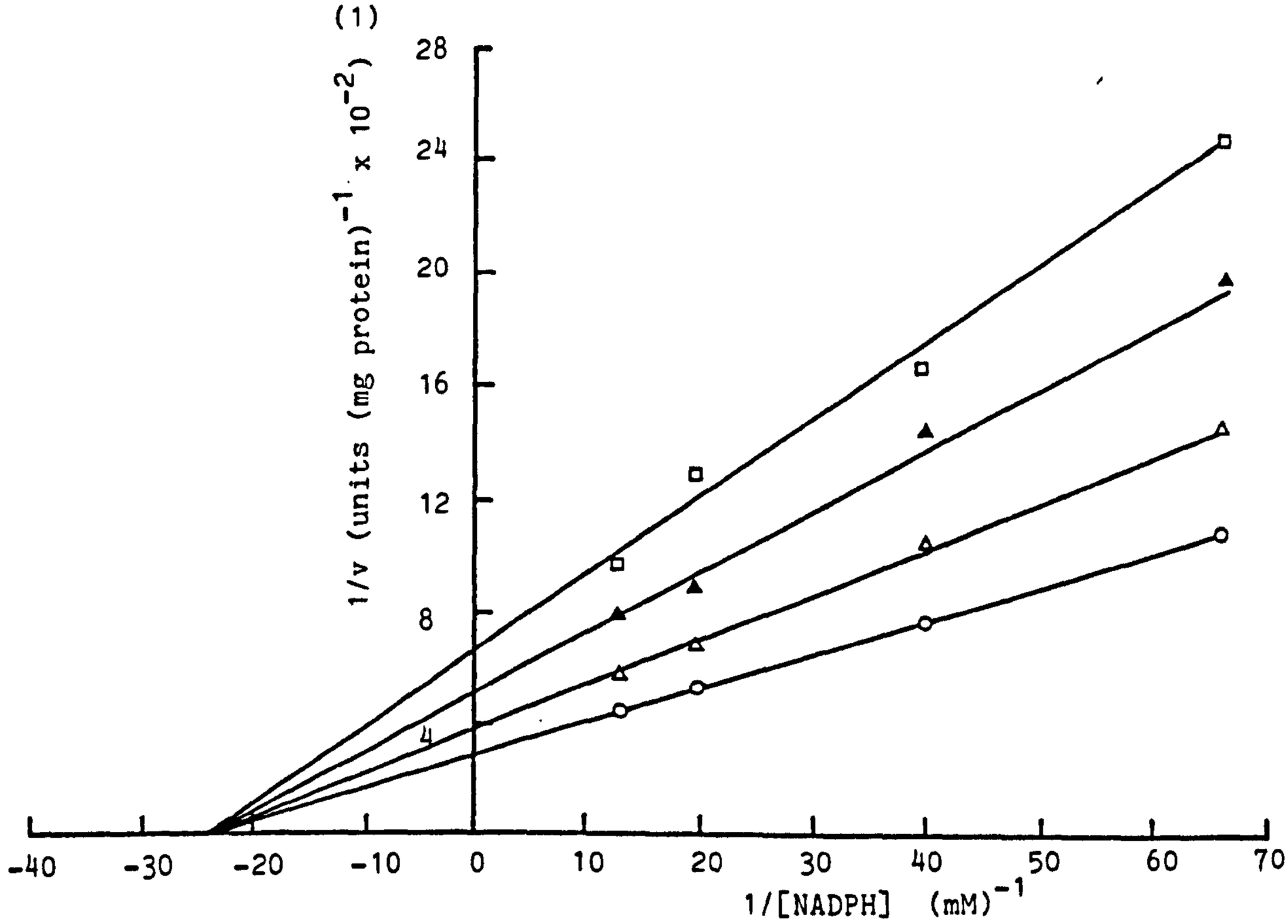
(○) 0; (△) 0.05; (▲) 0.10; (□) 0.15.

(1) Lineweaver-Burk transformation of data.

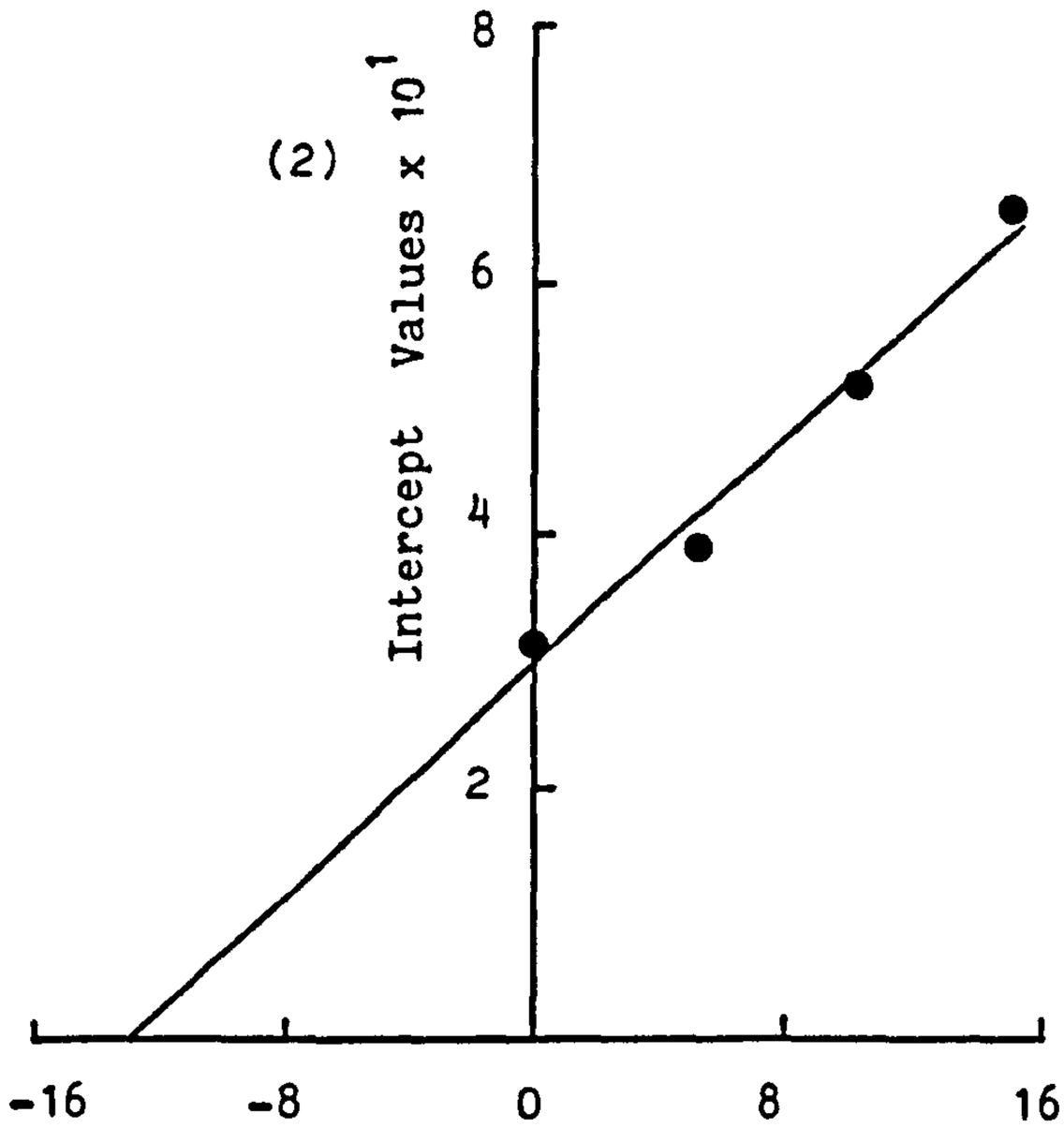
(2) Secondary re-plot of slope values obtained from data shown in (1).

(3) Secondary re-plot of intercept values obtained from data shown in (1).

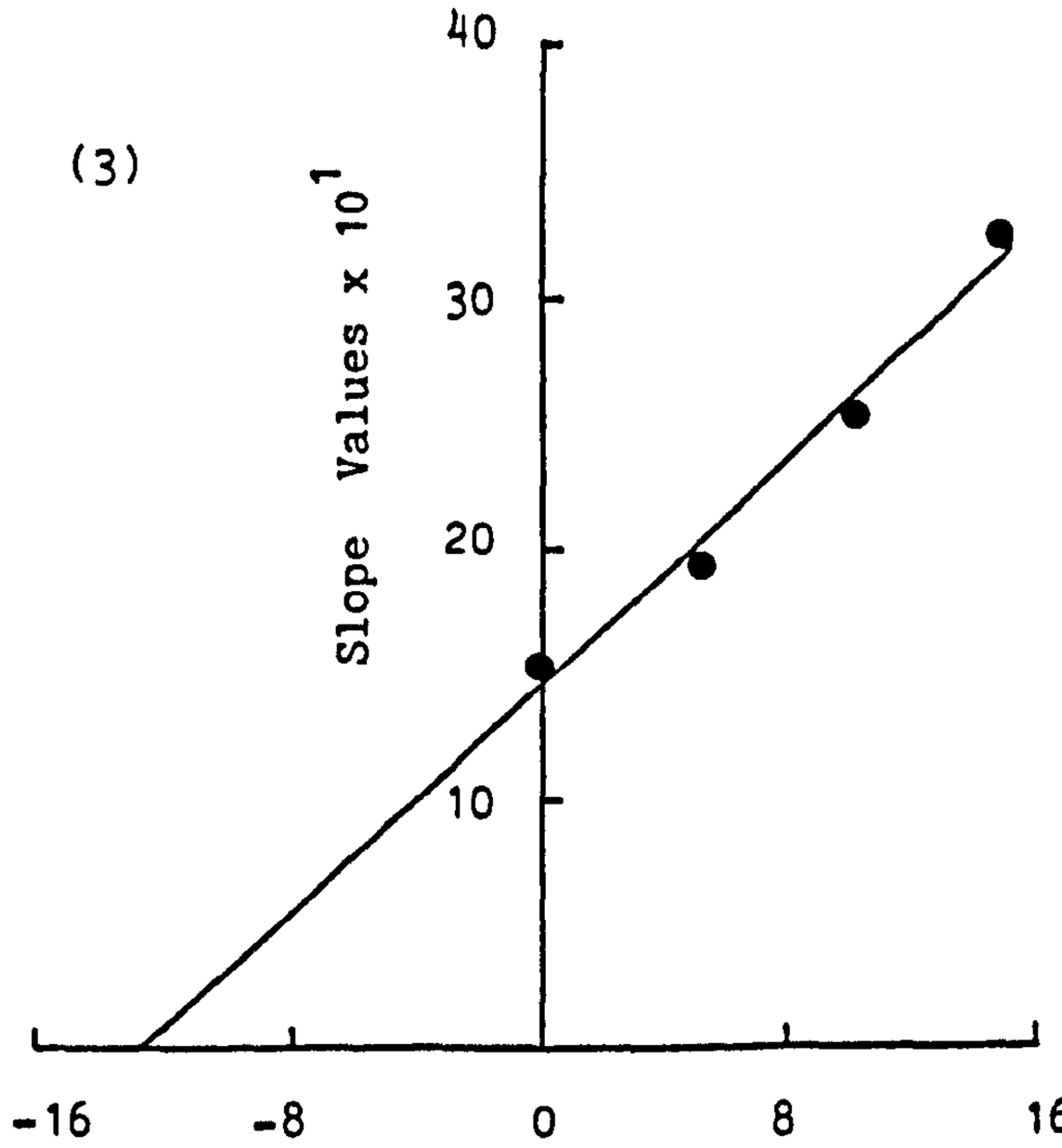
(1)


1/v (units (mg protein)$^{-1}$ x 10^{-2})
28
24
20
16
12
8
4
-40
-30
-20
-10
0
10
20
30
40
50
60
70
1/[NADPH] $(mM)^{-1}$

(2)


Intercept Values x 10^1
8
6
4
2
-16
-8
0
8
16
[D(-)-3-hydroxybutyrate (mM) x 10^2

(3)


Slope Values x 10^1
40
30
20
10
-16
-8
0
8
16
[D(-)-3-hydroxy - butyrate](mM) x 10^2

(Figure 6.9.2) and K_{ii} (Figure 6.9.3) determined in this case were both 0.4 mM.

Inhibition kinetic studies conducted in the presence of D(-)-3-hydroxybutyryl-CoA revealed that this molecule combined with a different enzyme form than either NADPH or acetoacetyl-CoA. If this was not the case competitive inhibition with respect to either acetoacetyl-CoA or NADP would have been observed. Since NADPH inhibition studies suggested that NADP is bound first by the enzyme and NADPH released last in the reaction sequence, D(-)-3-hydroxybutyryl-CoA appears to inhibit the enzyme by preventing the dissociation of NADP from the enzyme. This is consistent with a displacement of the equilibrium of the reaction in the direction of acetoacetyl-CoA formation.

Acetoacetyl-CoA reductase was also subject to substrate inhibition. This was demonstrated in Figure 6.10 where an increase in the concentration of acetoacetyl-CoA, above 20 μM at the two levels of NADPH tested, effected a progressive decrease in the velocity of the reaction catalysed by this enzyme. At both levels of NADPH, 50 % inhibition of enzyme activity was noted at 80 μM acetoacetyl-CoA. Although the site of inhibition of acetoacetyl-CoA with the enzyme reaction mechanism was not investigated further, substrate inhibition of this type is usually a consequence of the formation of an abortive reaction complex. In this case, the reaction complex E.acetoacetyl-CoA is possibly formed instead of the active complex E.NADP.acetoacetyl-CoA (see equation 6.4).

Figure 6.9 The Effect of D(-)-3-Hydroxybutyryl-CoA Concentration on the Initial Velocity of the Reaction Catalysed by Acetoacetyl-CoA Reductase, in the Presence of Fixed, Saturating NADP and Variable Acetoacetyl-CoA Concentration

Cuvettes contained in a total reaction volume of 1 ml (final concentration): Tris-HCl buffer (100 mM, pH 8.2); $MgCl_2$ (20 mM); NADPH (0.15 mM); acetoacetyl-CoA (2.0 - 7.0 μM); purified enzyme preparation (0.29 μg of protein).

Concentration of D(-)-3-hydroxybutyryl-CoA (mM):

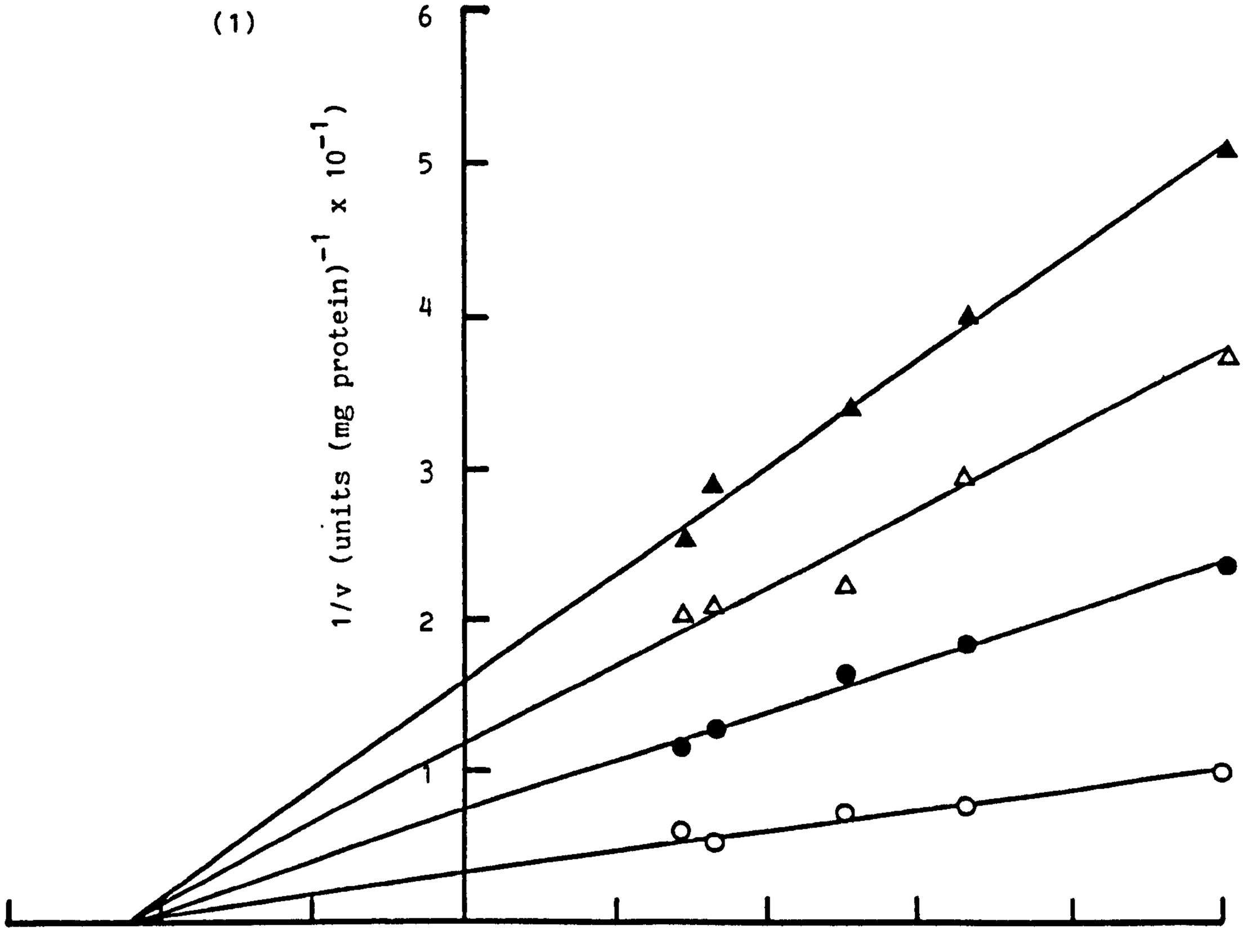
(○) 0; (●) 0.05; (△) 0.10; (▲) 0.15.

(1) Lineweaver-Burk transformation of data.

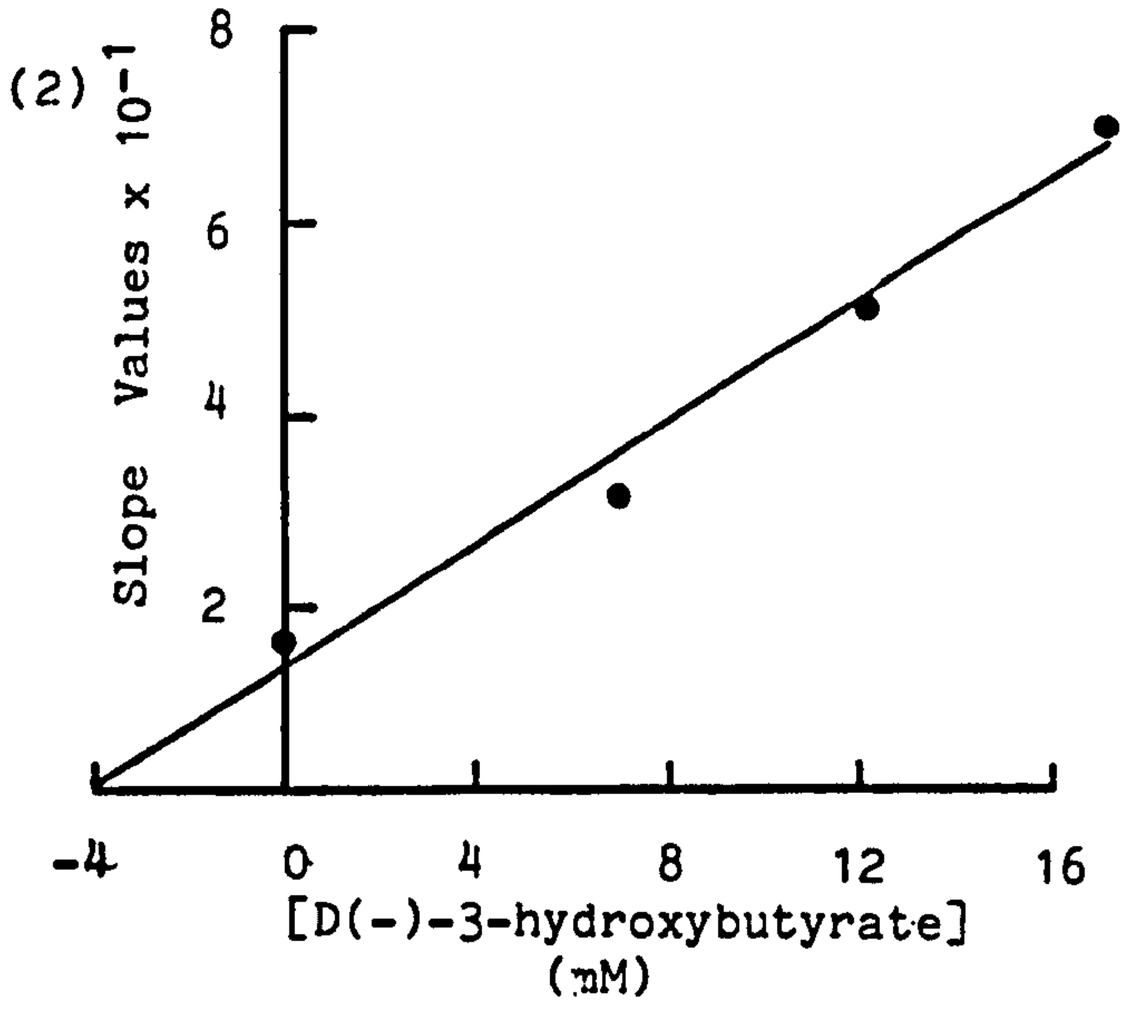
(2) Secondary re-plot of slope values obtained from data shown in (1).

(3) Secondary re-plot of intercept values obtained from data shown in (1).

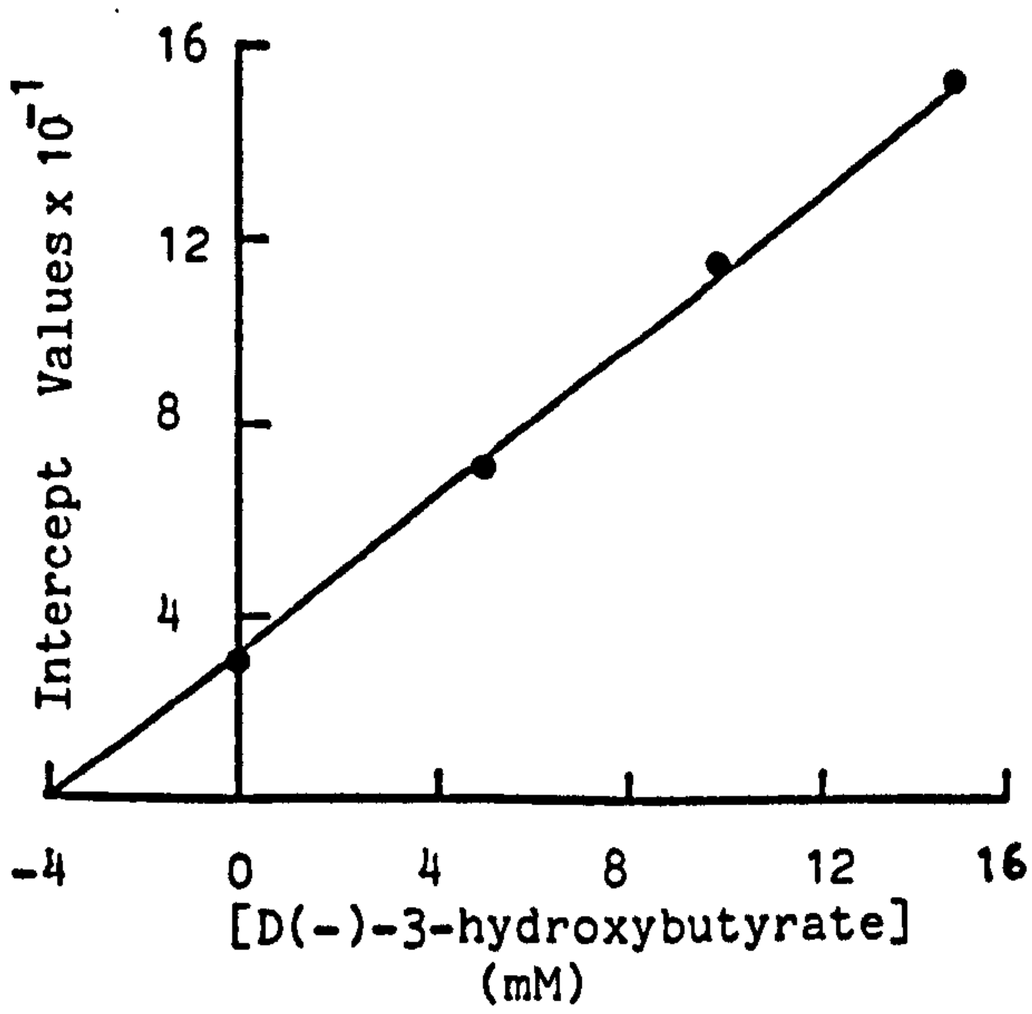
(1)


1/v (units (mg protein)$^{-1}$ x 10^{-1})
6
5
4
3
2
1
-3
-2
-1
0
1
2
3
4
5
1/[Acetoacetyl-CoA] (mM)$^{-1}$ x 10

(2)


Slope Values x 10^{-1}
8
6
4
2
-4
0
4
8
12
16
[D(-)-3-hydroxybutyrate] (mM)

(3)


Intercept Values x 10^{-1}
16
12
8
4
-4
0
4
8
12
16
[D(-)-3-hydroxybutyrate] (mM)

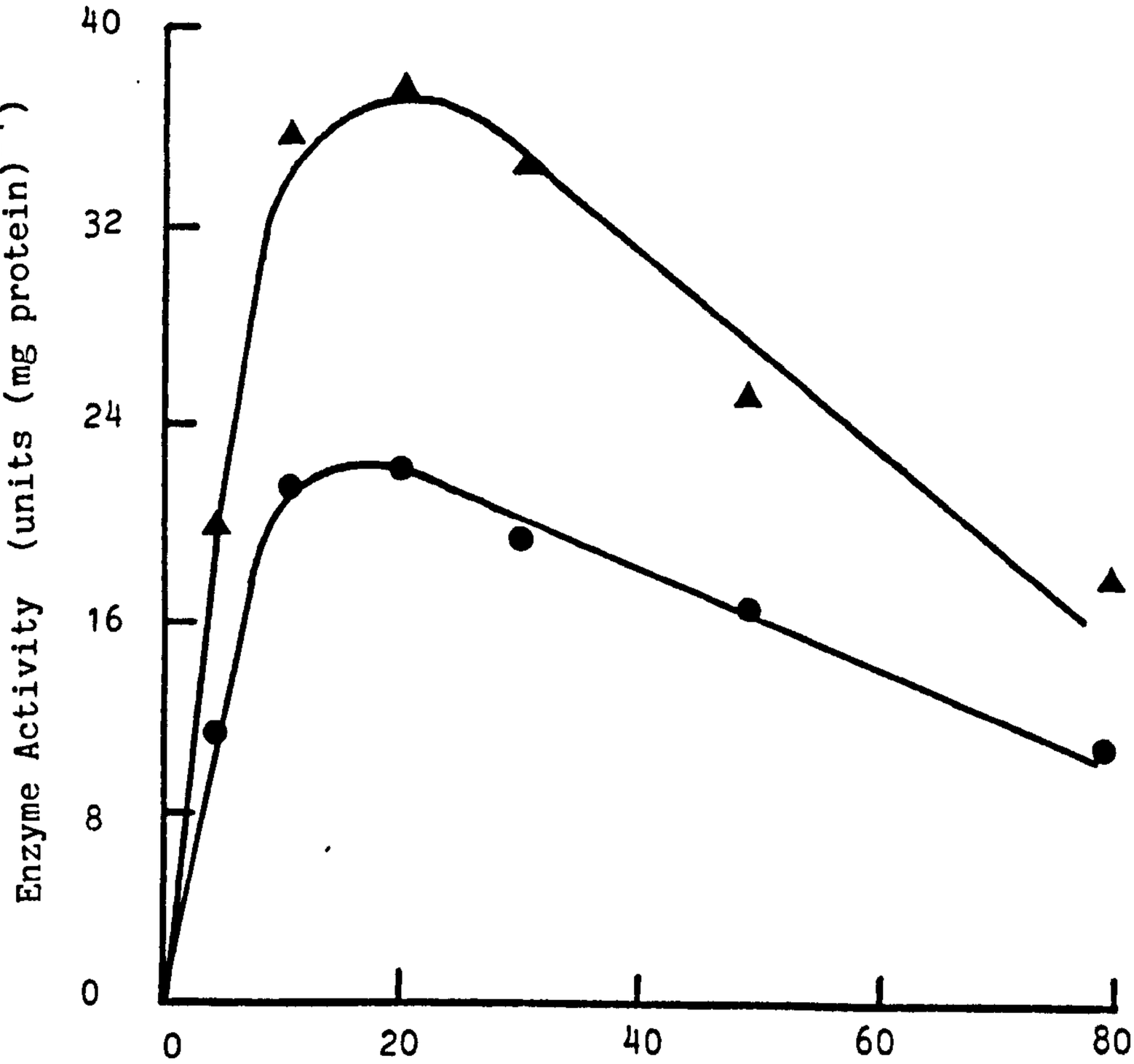


Figure 6.10 Effect of Increasing Concentration of Acetoacetyl-CoA on the Activity of Acetoacetyl-CoA Reductase from M. trichosporium OB3b

Cuvettes contained in a total reaction volume of 1 ml (final concentration): Tris-HCl buffer (100 mM, pH 8.2); $MgCl_2$ (20 mM); acetoacetyl-CoA (5 - 80 μM); purified enzyme preparation (0.29 μg of protein). Concentration of NADPH (mM): (●) 0.05; (▲) 0.1. The reaction was monitored at 303 nm as described in section 6.2.2.

6.3.6 EQUILIBRIUM CONSTANT OF ACETOACETYL-COA REDUCTASE

The equilibrium constant for the reaction catalysed by acetoacetyl-CoA reductase was determined in the direction of D(-)-3-hydroxybutyryl-CoA formation (equation 6.3)

$$\text{Acetoacetyl-CoA} + \text{NADP} \rightleftharpoons \text{D(-)-3-Hydroxybutyryl-CoA} + \text{NADPH} + H^+ \quad (6.3)$$

The equilibrium constant, K, was derived from the equation:

$$K = \frac{[\text{D(-)-3-hydroxybutyryl-CoA}]\ [\text{NADPH}]\ [H^+]}{[\text{acetoacetyl-CoA}]\ [\text{NADP}]}$$

K was determined to be 0.15×10^{-6} M at pH 7.5 indicating that the equilibrium of the reaction favours the formation of D(-)-3-hydroxybutyryl-CoA.

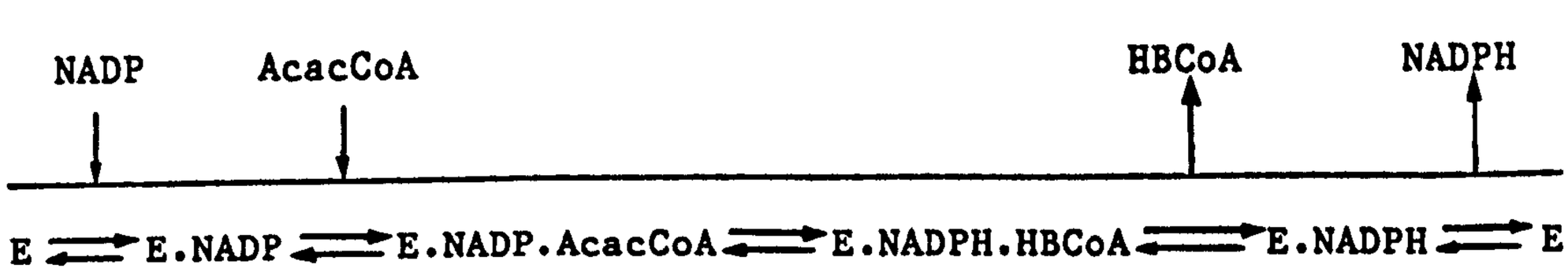
6.4 DISCUSSION

The discovery of a NADPH-dependant acetoacetyl-CoA reductase from M. trichosporium OB3b suggested that the pathway leading to the synthesis of D(-)-3-hydroxybutyryl-CoA from acetyl-CoA was probably the same as that indicated in the majority of PHB producing bacteria studied to date (Carr & Lascelles, 1961; Schindler, 1964; Kominek & Halvorson, 1965; Ritchie et al., 1971; Taylor & Anthony, 1976; Saito et al., 1977). That is, following the formation of acetoacetyl-CoA, by the action of beta-ketothiolase, D(-)-3-hydroxybutyryl-CoA is formed via a one step reduction of acetoacetyl-CoA. In this way the pathway operating in M. trichosporium OB3b differs from the one described in R. rubrum (Moskowitz & Merrick, 1969), where the sequential action of a dehydrogenase and two stereospecific dehydratases effected the same net synthesis of D(-)-3-hydroxybutyryl-CoA.

The method developed in this study for the purification of acetoacetyl-CoA reductase from M. trichosporium OB3b represents the first description of the use of triazine dye affinity chromatography in the purification of this enzyme from any source to date. The study highlighted the extremely high binding affinity of acetoacetyl-CoA reductase for the triazine dye conjugates tested. This in turn was reflected by the high level of purification achievable by resolving enzymes on the basis of their binding affinity alone. Although it is probably unfair to compare the efficiency of this scheme against those used to purify the same enzyme from other sources (Ritchie et al., 1971; Saito et al., 1977), it certainly represents a remarkable

example of the exploitation of triazine dye affinity chromatography for the highly specific fractionation of a cofactor dependant enzyme.

The study also provides the first detailed kinetic study on acetoacetyl-CoA reductase from any source. Product inhibition kinetics not only provide information on the possible regulation of an enzyme but also its reaction mechanism (Cleland, 1967). In this case the kinetic data was consistent with an ordered Bi-Bi mechanism such as that highlighted previously by 3-HBD (section 3.3) and many pyridine nucleotide dehydrogenases (Tan et al., 1975; Dalziel, 1975). On the basis of this data a possible reaction mechanism for the enzyme is proposed as follows (equation 6.4):



...(6.4)

Abbreviations: E, acetoacetyl-CoA reductase; AcacCoA, acetoacetyl-CoA; HBCoA, D(-)-3-hydroxybutyryl-CoA.

A comparison of the properties of acetoacetyl-CoA reductase from M. trichosporium OB3b with those obtained from the detailed studies on two other PHB producing bacteria, namely, Z. ramigera I-16-M (Saito et al., 1977) and A. beijerinkii (Ritchie et al., 1971; Senior & Dawes, 1973) shows that a number of properties are common to all these enzymes. In the first instance, the K_m of each enzyme for acetoacetyl-CoA is extremely low (in the range 1 - 8.3 x 10^{-6} M); acetoacetyl-CoA also acts as a substrate inhibitor under certain

conditions (typically at concentrations 10 μM). Each of the three enzymes were NADPH-linked and were not susceptible to inhibition apart from the substrate and reaction products of the enzyme. Furthermore, where measured (Ritchie et al., 1971; this study), the equilibrium constant of acetoacetyl-CoA reductase favoured the formation of D(-)-3-hydroxybutyryl-CoA ($K = 3.54 \times 10^{-10}$ M at pH 7.5).

By way of contrast, however, the methanotrophic enzyme was susceptible to a double product inhibition (NADP and D-(-)-3-hydroxybutyryl-CoA), whereas a similar inhibition was not reported in the case of either of the Azotobacter or the Zoogloea enzymes. The significance of these differences are difficult to ascertain categorically and any conclusions drawn must remain speculative. Nevertheless, as highlighted below, the kinetic data obtained from the methanotrophic acetoacetyl-CoA reductase suggests that it plays an important role in the regulation of PHB synthesis. Furthermore, the data also suggests that careful regulation of its activity is critical to PHB mobilisation.

The kinetic study on beta-ketothiolase from M. trichosporium OB3b (chapter 5) suggested that the first committed step towards PHB synthesis was controlled by the acetyl-CoA/CoASH ratio; the study on the regulation of acetoacetyl-CoA reductase from this organism essentially extends this reasoning. Since the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA is known to be more favourable thermodynamically in the direction of acetoacetyl-CoA cleavage, the regulation and properties of the enzyme catalysing the next step in the reaction sequence is probably of equal importance in

the regulation of PHB synthesis. Efficient metabolism of acetoacetyl-CoA is required in order to prevent the beta-ketothiolase reaction from reaching equilibrium. Certainly, the acetoacetyl-CoA reductase from this organism is ideally suited to its role in PHB synthesis. In the first instance it possesses an extremely low K_m for acetoacetyl-CoA (4.5×10^{-6} M); this compares with a K_m value of 30×10^{-6} M obtained for beta-ketothiolase in this study (section 6.3). Also the equilibrium constant of the enzyme reaction favours the formation of D(-)-3-hydroxybutyryl-CoA. Under favourable conditions pertaining to PHB synthesis one might therefore expect the intracellular concentration of acetoacetyl-CoA to be extremely low, since Cleland (1970) suggests that the K_m value of an enzyme reflects the intracellular concentration of its substrate.

The role played by NADP, acetoacetyl-CoA and D(-)-3-hydroxybutyryl-CoA inhibition of acetoacetyl-CoA reductase in the regulation of PHB synthesis remains speculative in the absence of in vivo metabolite levels during polymer metabolism. The kinetic data suggests that these compounds rank in the order of acetetoacetyl-CoA > NADP > D(-)-3-hydroxybutyryl-CoA in terms of their potency of inhibition of the enzyme. Under conditions of PHB synthesis one might therefore expect the concentration of these metabolites to be low in order to derive the maximum catalytic capacity of the enzyme for the production of D(-)-3-hydroxybutyryl-CoA. Under conditions where either of these metabolites might accumulate, the concerted inhibition on beta-ketothiolase might be sufficient to arrest polymer synthesis. Whether concerted inhibition of the nature described here regulates the level of polymer accumulation is an interesting, but speculative

proposition. Certainly the problem arises during PHB mobilisation of the wasteful cycling of acetoacetyl-CoA, formed by acetoacetyl-CoA synthetase, via D(-)-3-hydroxybutyryl-CoA formation. However, during PHB mobilisation one might expect that the intracellular concentration of acetoacetyl-CoA to be high with subsequent inhibition of the reductase. Since the inhibition of acetoacetyl-CoA reductase is dependant upon the intracelluar concentration of NADPH, under conditions of PHB mobilisation the acetoacetyl-CoA/NADPH ratio should ideally be high. If this were the case, it would mirror conditions thought optimal for the mobilisation of PHB via 3-HBD, i.e., a high ratio of oxidised/reduced pyridine nucleotide.

C H A P T E R S E V E N

PHB SYNTHESIS AND *IN VIVO* METABOLITE STUDIES IN *M. TRICHOSPORIUM* OB3B

7.1 SUMMARY

The nutrient limitation responsible for the accumulation of PHB in methanol-grown M. trichosporium OB3b is assessed by continuous culture experiments. In conjunction with this study, a method is described for the simultaneous quantification of the CoA compounds shown previously to act as effectors of beta-ketothiolase (section 5.3.6) and acetoacetyl-CoA reductase (section 6.3.5). This preliminary investigation forms the basis of a discussion on the possible control of PHB metabolism in M. trichosporium OB3b. This accounts for both the kinetic and regulatory data obtained from studies on the purified enzymes associated with PHB metabolism and in vivo metabolite quantification during the synthesis and mobilisation of this polymer. The wider implications of this study on the intermediary metabolism of this organism is also assessed.

7.2 INTRODUCTION

Despite numerous reports on PHB accumulation in a wide variety of organisms, during both batch growth (Macrae & Wilkinson, 1958a; Repaske & Repaske, 1976; Tezuka et al., 1980; Suzuki et al., 1986a) and continuous culture (Wilkinson & Munroe, 1967; Senior & Dawes, 1972), our present knowledge on the biochemical regulation of PHB metabolism is still somewhat limited and speculative. The kinetic and regulatory data derived from studies on key enzymes associated with PHB metabolism have served to highlight the significance of specific metabolites in their regulation (Senior & Dawes, 1973; Saito et al.,

1977; Nishimura et al., 1978; Nakada et al., 1981). Nevertheless, in the absence of corroborative in vivo metabolite measurements during PHB synthesis/mobilisation it is difficult to rationalise the importance of such information in isolation. Kinetic data obtained from three soluble enzymes associated with PHB metabolism in M. trichosporium OB3b served to highlight the relative significance of specific metabolites on the regulation of these enzymes in vivo. These compounds were identified as follows: acetyl-CoA, acetoacetyl-CoA, D(-)-3-hydroxybutyryl-CoA, CoASH, the ratios of NADP/NADPH and that of NAD/NADH. During this study, however, the significance of the CoASH compounds only were examined with respect to their role in the regulation of polymer metabolism.

7.3 PHB ACCUMULATION IN M. TRICHOSPORIUM OB3B DURING BATCH CULTURE ON METHANOL

Prior to batch growth studies on PHB accumulation in M. trichosporium OB3b it was necessary to adapt this organism to growth on methanol. This was achieved by a modification of the method first described by Hou et al. (1979). This comprised the following: Centre well flasks, containing NSM minimal salts medium (50 ml) with methanol (1 ml) in the centre well, were inoculated with methane-grown colonies from NSM agar plates. By further addition of filter sterilised $NaHCO_3$ (0.25 % (v/v) final concentration) to the growth medium, the organism adapted to growth on methanol in a reproducable fashion after 48 hours. Attempts to adapt cultures to grow on methanol without the prior addition of $NaHCO_3$, as described by Hou et al. (1979), were characterised by a considerable and variable lag phase

before any visible growth was noted; this lag normally lasted from four to ten days.

Since the generation of new cell material in this organism is dependant upon the operation of the serine pathway, and the incorporation of carbon, as CO_2 via phosphoenol pyruvate carboxylase, the possibility that $NaHCO_3$ addition to the growth medium furnishes these requirements during the early phase of growth appears to be the most favourable conclusion.

Although M. trichosporium OB3b was routinely maintained in liquid culture using nitrate minimal salts medium and methanol (0.5 % (v/v)), numerous attempts to culture this organism on solid media containing methanol was unsuccessful. Different strategies included the incorporation of methanol directly into the solid media and also presentation in the vapour phase.

The accumulation of PHB during batch culture of M. trichosporium OB3b on methanol was studied at two concentrations. When the organism was grown on 0.4 % (v/v) methanol (Figure 7.1.1) the formation of PHB occurred predominantly towards the end of the exponential phase of growth, reaching a maximum of 11.6 % (w/w) dry weight. Following exhaustion of methanol from the growth medium, the biosynthesis of polymer ceased. Within 30 hours of this event the PHB content of the organism had decreased to 1 % (w/w) dry weight. Somewhat surprisingly, no significant decrease in culture dry weight was noted during this period. Whether this can be accounted for by an increase in cell numbers was not ascertained. Clearly, under these circumstances, PHB

appears to act as a reserve material capable of furnishing the carbon requirements of the cell under conditions where the primary carbon source had been exhausted.

When the organism was grown in a medium containing 1.0 % (v/v) methanol (Figure 7.1.2) the pattern of PHB accumulation was largely similar to that described for growth on 0.4 % (v/v) methanol. However, on reaching a maximum deposition of 19.2 % (w/w) dry weight, there was no significant decrease in the content of polymer during the subsequent time course of the study; the residual concentration of methanol remaining in the growth medium was 0.26 % (v/v). This suggests that the culture is limited by some factor other than carbon. The possibility that the large quantities of PHB present in this organism, following cessation of growth, might ultimately contribute towards the energetic requirements of spore formation, is an interesting proposition. However, this was not examined further in this study.

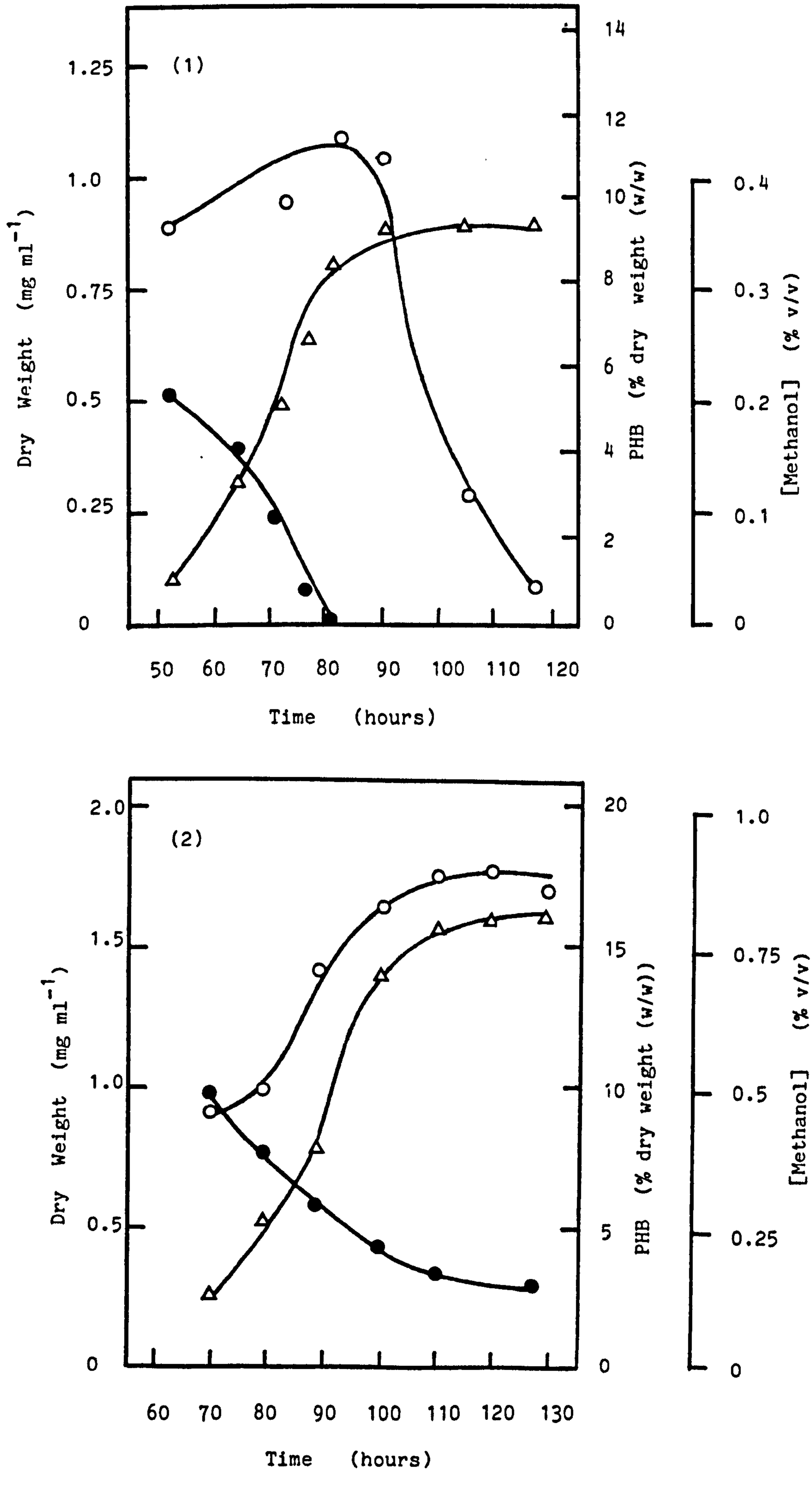
The information revealed by batch growth experiments on M. trichosporium OB3b was limited. It was impossible at this stage to define the growth limiting nutrient responsible for PHB accumulation. The next section attempts to resolve this problem by use of chemostat growth growth analysis.

Figure 7.1 Growth and PHB Content of M. trichosporium OB3b During Batch Culture on Methanol

For details on the growth media see section 2.2. (○) PHB content; (△) bacterial dry weight; (●) methanol concentration in culture supernatant.

(1). Limiting methanol (0.4 % v/v)

(2). Excess of methanol (1.0 % v/v)


(1)
Dry Weight (mg ml^{-1})
PHB (% dry weight (w/w))
[Methanol] (% v/v)
1.25
1.0
0.75
0.5
0.25
0
14
12
10
8
6
4
2
0
0.4
0.3
0.2
0.1
0
50 60 70 80 90 100 110 120
Time (hours)
(2)
Dry Weight (mg ml^{-1})
PHB (% dry weight (w/w))
[Methanol] (% v/v)
2.0
1.5
1.0
0.5
0
20
15
10
5
0
1.0
0.75
0.5
0.25
0
60 70 80 90 100 110 120 130
Time (hours)

7.4 PHB ACCUMULATION IN M. TRICHOSPORIUM OB3B DURING CONTINUOUS CULTURE ON METHANOL

One of the main objectives of the batch growth experiments on M. trichosporium OB3b was to assess the extent of polymer accumulation in this organism. The purpose of the next phase of the study was to determine the nutrient limitation responsible for PHB accumulation, such that one might switch from low to high PHB production and vice versa by inclusion or omission of a single nutrient in the growth medium.

Since PHB accumulation has been shown, in the majority of cases, to be attributable to either a nitrogen (Sonnleitner et al., 1979) or an oxygen limitation (Senior et al., 1972) of growth, the effects of both types of nutrient stress on PHB synthesis in M. trichosporium OB3b were investigated by continuous culture experiments.

7.4.1 The EFFECT OF OXYGENATION ON PHB SYNTHESIS IN M. TRICHOSPORIUM OB3B DURING CONTINUOUS CULTURE ON METHANOL

This experiment was devised to assess the effect of oxygen limitation on the dry weight and PHB content of M. trichosporium OB3b, during continuous culture on methanol (0.4 % (v/v)). At the methanol feedstock concentration used in this experiment, with a dilution rate of 0.04 h^{-1}, the PHB content of this organism was typically less than 2 % (w/w) dry weight. The steady state cell density (0.94 g (dry weight) l^{-1}), was close to that found in shake flask culture (1.05 g (dry weight) l^{-1}).

The organism was grown in the steady state at a dissolved oxygen tension (D.O.T.) of 50 % (100% = air saturation under these conditions of temperature and ionic strength) and at a time zero, the air inlet controller was adjusted to give a D.O.T. of 0 %. PHB content and cell density was monitored for approximately 2 generations (Figure 7.2). From the data obtained it was evident that oxygen limitation was not responsible for PHB accumulation in this organism. The dry weight of the culture fell during the decrease in D.O.T. and re-established a new steady state level corresponding to 0.52 g (dry weight) 1^{-1}. The PHB content of the culture remained at a basal level (less than 2 % (w/w) dry weight) throughout the experiment.

7.4.2 THE EFFECT OF NITROGEN LIMITATION ON PHB SYNTHESIS IN M. TRICHOSPORIUM OB3B DURING CONTINUOUS CULTURE ON METHANOL

The effect of nitrogen limited growth on the PHB content of a continuous culture of M. trichosporium OB3b was assessed in a similar fashion to that described in the previous experiment (section 7.4.1). The organism was grown at a dilution rate of 0.04 h^{-1}, a methanol feedstock concentration of 0.4 % (v/v) and a D.O.T. of 50 %. At a time zero, the nitrogen (NH_4) content of the minimal salts inlet medium was reduced by 50 %; the PHB content and the cell density of the culture was monitored for approximately 2 generations. Figure 7.3 shows that a fall in cell density of the culture paralleled an increase in the production of PHB; a maximum deposition of 13.65 % (w/w) dry weight was recorded in this experiment.

As a consequence of this study, growth conditions were

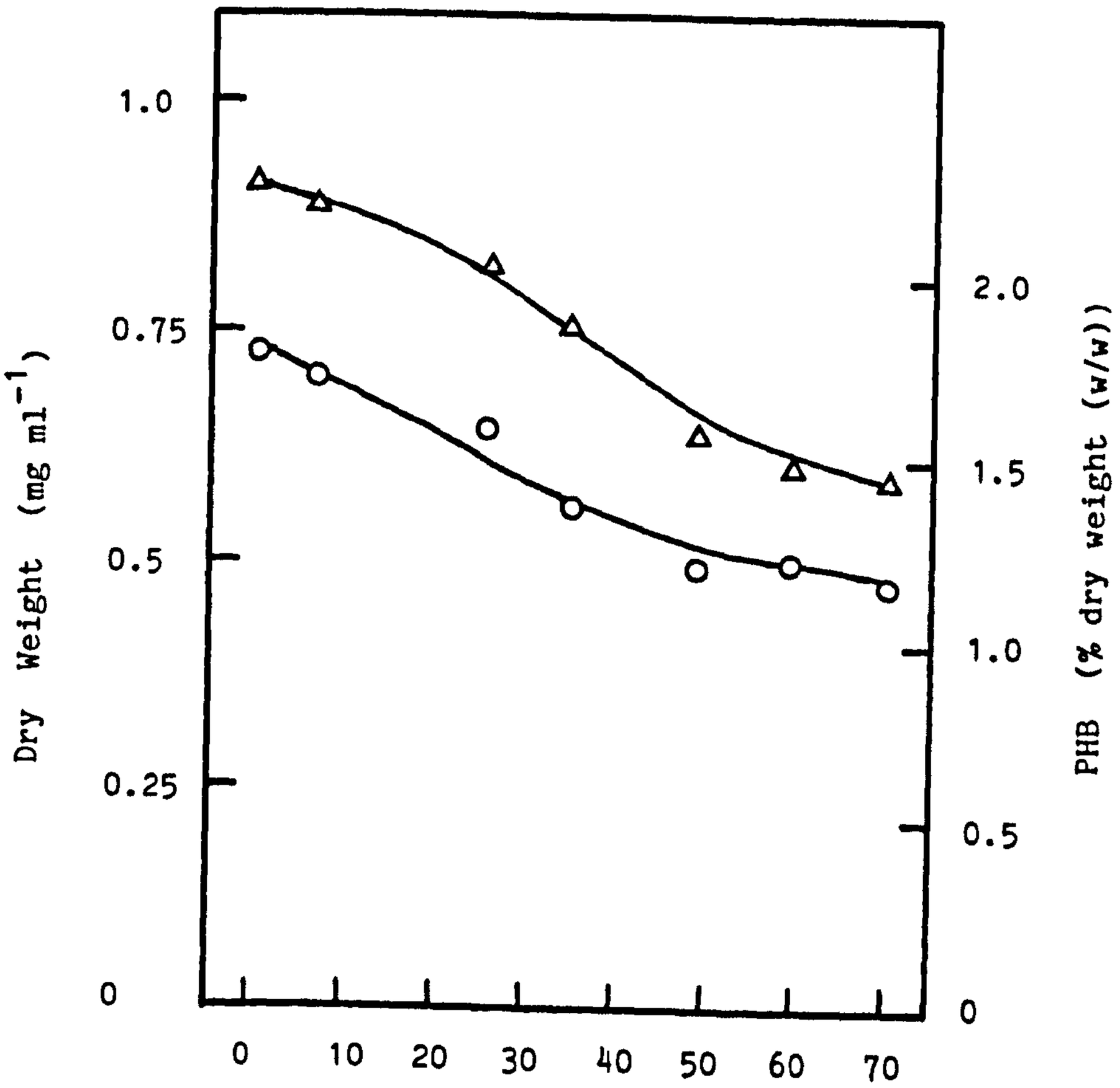


Figure 7.2 Effect of Oxygen Limitation on the Growth and PHB Content of M. trichosporium OB3b During Continuous Culture on Methanol (0.4 % v/v)

For experimental details see the text.

(○) PHB content; (△) bacterial dry weight.

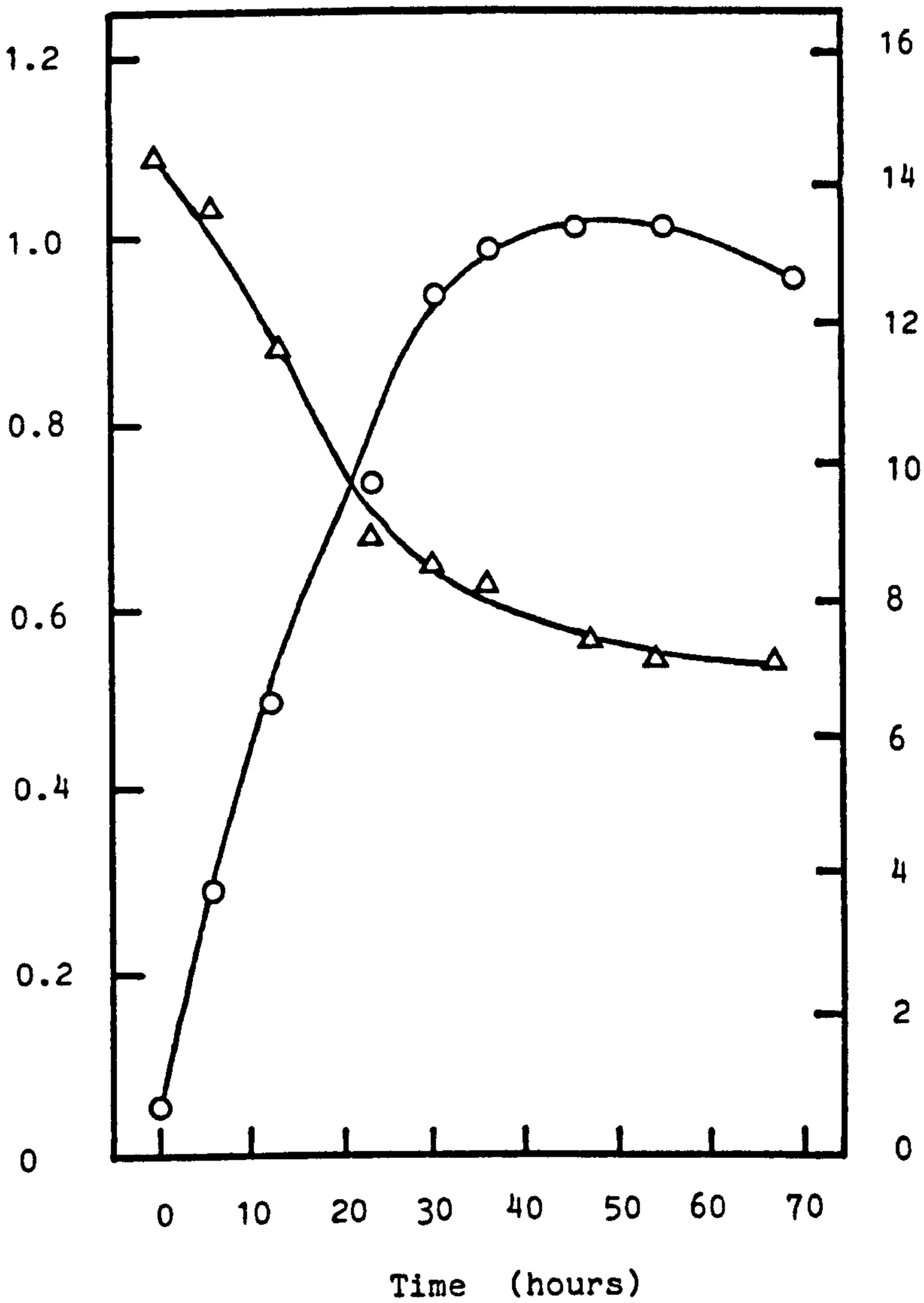


Figure 7.3 Effect of Nitrogen Limitation on the Growth and PHB content of M. trichosporium OB3b During Continuous Culture on Methanol (0.4 % v/v)

For experimental details see the text.

(○) PHB content; (△) bacterial dry weight.

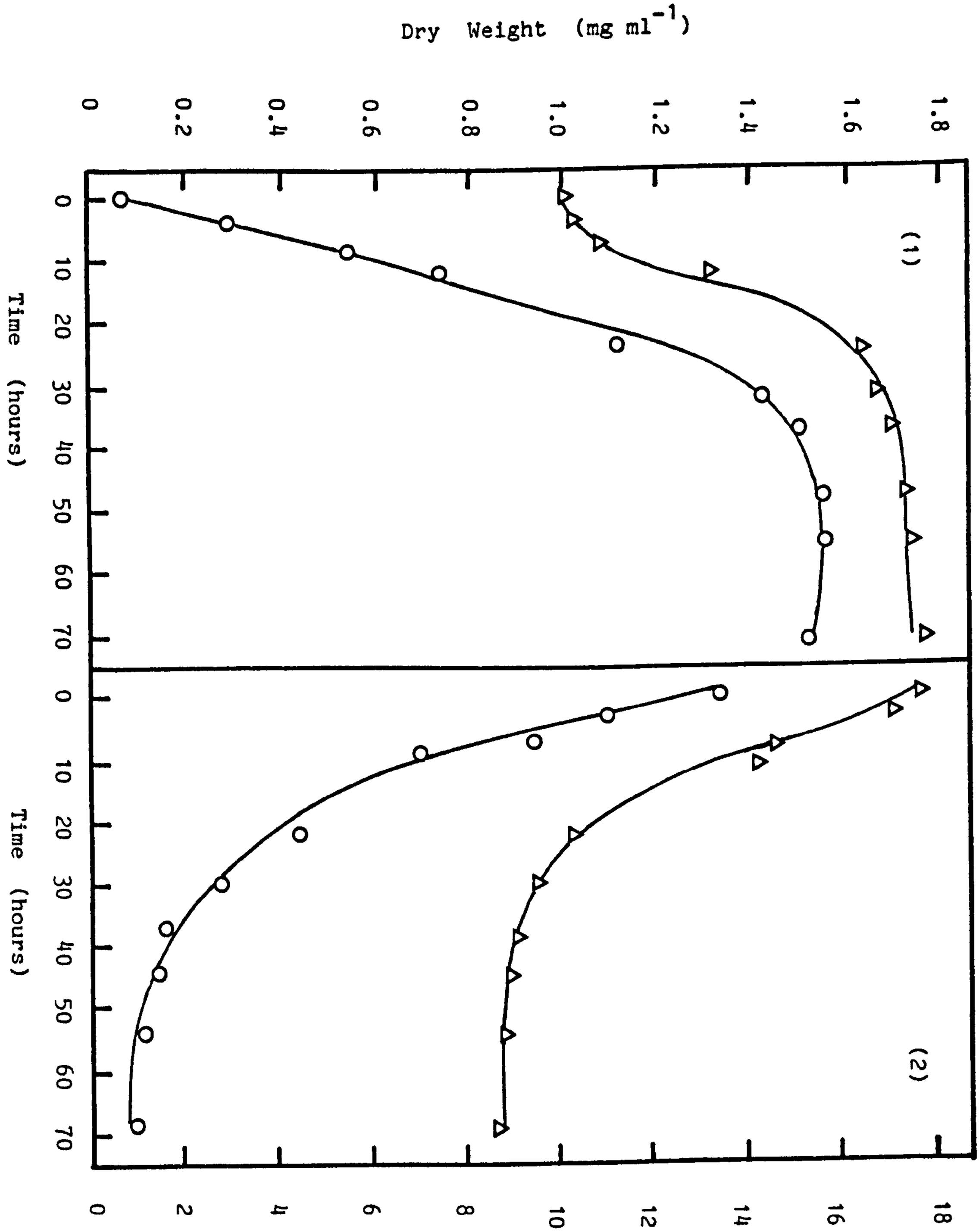
established where one might induce PHB synthesis and subsequently its mobilisation. For this purpose, switching from a methanol feedstock concentration of 0.4 % (v/v) to 1.0 % (v/v), at a constant nitrogen and oxygen level, and vice versa, proved satisfactory. An increase in the inlet methanol concentration to the fermenter, from 0.4 % (v/v) to 1.0 % (v/v) resulted in an increase in the dry weight of the culture from 1.0 to 1.82 g (dry weight) l^{-1} with a corresponding increase in PHB content from 0.64 to 15.6 % (w/w) dry weight (Figure 7.4.1). During the switch from 1.0 % (v/v) to 0.4 % (v/v) methanol concentration a reversal in this trend resulted (Figure 7.4.2). Under these conditions the culture is effectively switching between nitrogen excess and nitrogen limitation on growth. Although other nutrient(s) limitations may induce PHB synthesis in this organism, they were not examined in this study.

The change in dry weight and intracellular PHB concentration during the methanol transition experiments indicated above were mirrored by changes in the levels of three enzymes associated with PHB metabolism, 3-HBD, beta-ketothiolase and acetoacetyl-CoA reductase. During the change from 0.4 % (v/v) to 1.0 % (v/v) methanol feedstock concentration each enzyme was induced approximately 3-fold above basal levels (Figure 7.5.1). The pattern of induction was similar in each case; maximum expression of activity was noted after 20 hours (approximately 1 cell generation). One slightly anomolous result in this study was the induction of 3-HBD during a time period characterised by PHB synthesis. Whether this affords the organism the capability to quickly reutilise PHB once it is synthesised is a speculative interpretation of the information. Nevertheless, the

Figure 7.4 Effect of Transition From Methanol Limitation (0.4 % v/v) to Methanol Excess (1.0 % v/v), and Vice Versa, on the Content of PHB in a Continuous Culture of M. trichosporium OB3b

(1) Transition from 0.4 % (v/v) to 1.0 % (v/v) methanol.
(○) PHB content; (△) bacterial dry weight.

(2) Transition from 1.0 % (v/v) to 0.4 % (v/v) methanol.
(○) PHB content; (△) bacterial dry weight.


Dry Weight (mg ml^{-1})
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
(1)
Time (hours)
0
10
20
30
40
50
60
70
(2)
Time (hours)
0
10
20
30
40
50
60
70
0
2
4
6
8
10
12
14
16
18
PHB (% dry weight (w/w))

increase in activity of both beta-ketothiolase and acetoacetyl-CoA reductase during this period fits the role of these enzymes in the pathway of PHB synthesis.

During PHB mobilisation (Figure 7.5.2) the level of activity of both beta-ketothiolase and acetoacetyl-CoA reductase fell whereas, that of 3-HBD remained constant. Presumably, the change in pattern of enzyme activities, during a period of PHB mobilisation, relates to the degree of participation of each enzyme in this pathway.

During the continuous culture experiments on M. trichosporium OB3b, certain observations were made which were critical to the success of each experiment. Attempts to introduce the switch from a methanol feedstock concentration of 0.4 % (v/v) to 1.0 % (v/v) at a dilution rate higher than 0.05 h^{-1} resulted in rapid washout of the culture. Furthermore, if, during the transition period, the culture pH was not maintained by automatic alkali addition the pH fell to 5.0. At this lower pH the culture was not viable and did not re-establish itself when shifted back to batch culture.

The metabolite responsible for this dramatic drop in pH was not identified but it might be due to the build up of formic acid. Challenging the culture with a relatively high local concentration of methanol might have resulted in its rapid oxidation to formate to prevent the accumulation of the former to toxic levels. Under conditions of lack of pH control formate build up might inhibit growth if the organism produces this quicker than it can metabolise it by way of the serine pathway. Instances where formate has been shown to

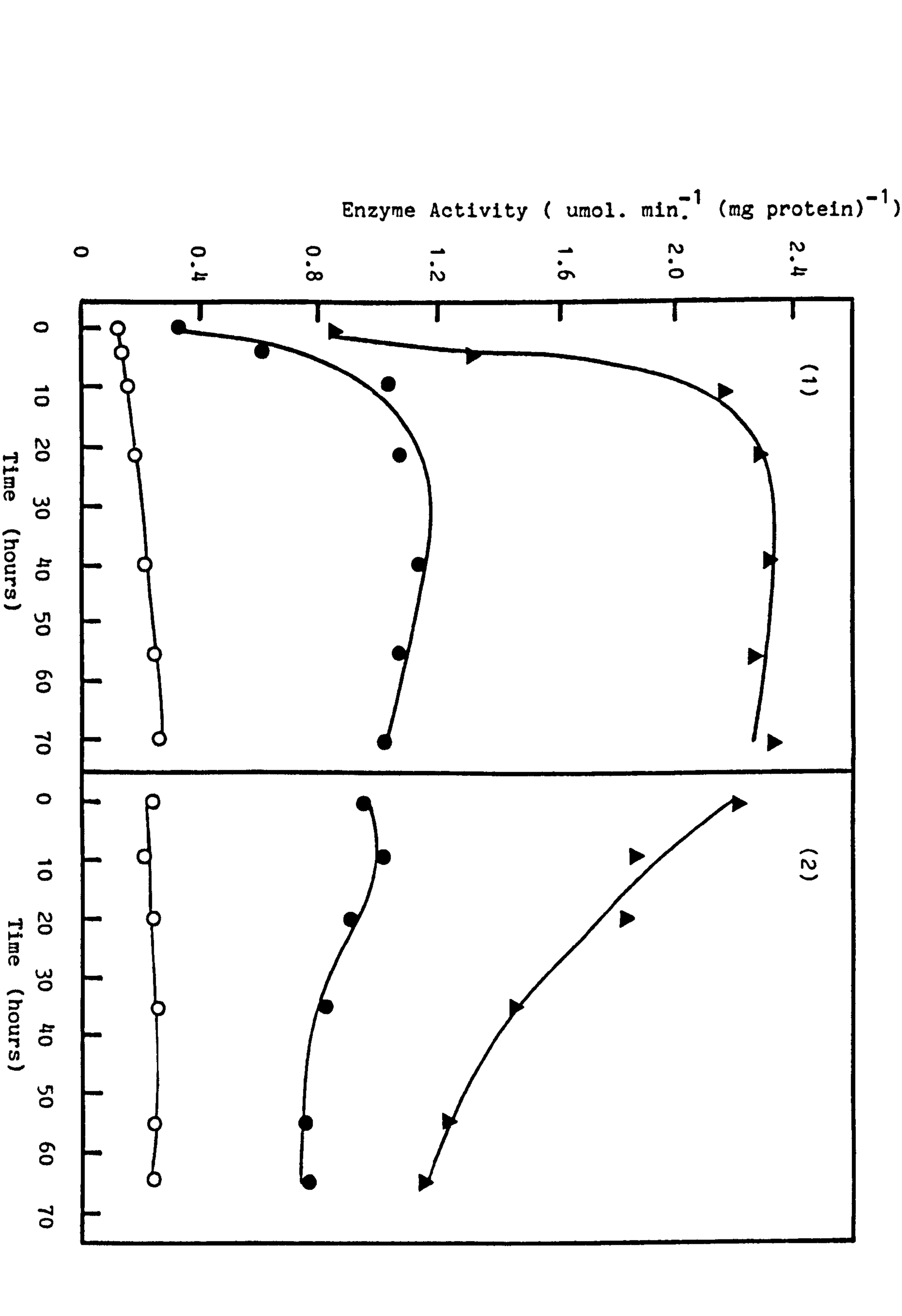
Figure 7.5 Effect of Transition From Methanol Limitation (0.4 % v/v) to Methanol Excess (1.0 % v/v), and Vice Versa, on the Expression of Enzyme Activity in a Continuous Culture of M. trichosporium OB3b.

For experimental details see the text. (O) 3-HBD;

(●) acetoacetyl-CoA reductase; (▲) beta-ketothiolase.

(1). Transition from 0.4 % (v/v) to 1.0 % (v/v) methanol.

(2). Transition from 1.0 % (v/v) to 0.4 % (v/v) methanol.


Enzyme Activity (umol. min.-1 (mg protein)-1)
0
0.4
0.8
1.2
1.6
2.0
2.4
(1)
(2)
Time (hours)
0
10
20
30
40
50
60
70

inhibit bacterial growth have been cited. Inhibition of methylotrophic growth by formate has been reported for Candida boidinii (Pilat & Prokop, 1975), although Methylocystis parvus OBBP was inhibited by high (2 % w/v) concentrations of this metabolite. Inhibition of growth by formic acid, rather than formate, has been reported during the continuous culture of of Hansenula polymorpha on methanol under oxygen limitation. The growth substrate also accumulated in this case, possibly by inhibition of energy transfer and utilisation or by the disintegration of phospholipid sequences in the cell membrane (Pilat & Prokop, 1976).

7.5 IN VIVO METABOLITE STUDIES ON M. TRICHOSPORIUM OB3B DURING CONTINUOUS CULTURE ON METHANOL

7.5.1 INTRODUCTION

Newsholme (1965, 1967) suggested a four stage approach to the analysis of control mechanisms related to intermediary metabolism. First, the enzymes involved in metabolic regulation are identified; second, their kinetic properties are investigated in detail; third, the kinetic properties are used to postulate a hypothesis of metabolic control; and fourth, the hypothesis is tested and is modified as necessary.

The first stage of this thesis has dealt with the first three requirements of a thorough study on metabolic regulation; this section is concerned with the fourth stage and involves the development of a methodology for the quantification of specific in vivo metabolites

during PHB metabolism in M. trichosporium OB3b. These compounds include acetoacetyl-CoA, acetyl-CoA, D(-)-3-hydroxybutyryl-CoA and CoASH. Furthermore, with the potential to simultaneously detect succinyl-CoA by this technique, information on the activity of the TCA cycle might also be monitored under these conditions.

7.5.2 THE SEPARATION AND QUANTIFICATION OF SHORT-CHAIN COA COMPOUNDS BY REVERSE-PHASE HPLC UTILISING UV DETECTION

The method used for the separation and quantification of short-chain CoA compounds was performed essentially as that described by King and Reiss (1985), with minor modifications to the gradient programme used in the elution of these compounds (see section 2.9). In this way, a solution of 6 CoA standards, relevant to this study, was separated in 25 min by reverse-phase chromatography using a 3-μM, C-18 column. A typical elution chromatogram of a standard solution of these compounds is shown in Figure 7.6.

A problem not uncommon to gradient separations of this type is baseline shift which can be caused from UV-absorbing impurities present in the buffer salts. Also, in view of the high sensitivity of detection employed during the quantification the of CoA compounds in this study, it was necessary to pre-purify the buffer salts. This was achieved by passing the stock phosphate buffer solution through tandem columns of ion-exchangers (AG 1-X8 and Chelex 100) as described previously (Reiss et al., 1984). The inclusion of an in-line column of activated charcoal served to remove traces of styrene-divinyl-benzene

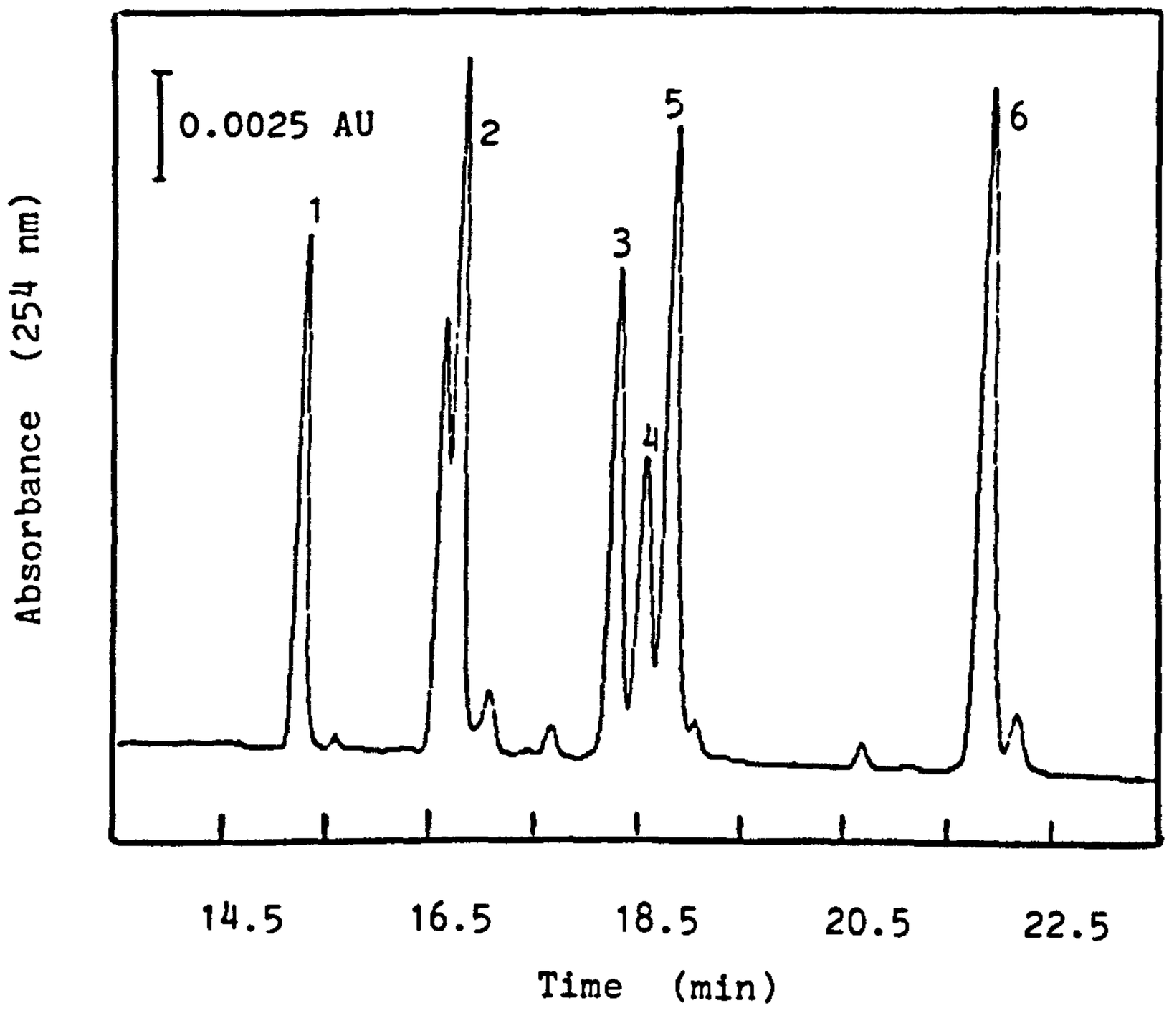


Figure 7.6 Chromatographic Separation of CoA Standards by Reverse Phase HPLC.

Column, 3 μM, C-18, 7.5 cm x 4.6 mm; temperature, 30°C; detector sensitivity, 0.05 AUFS. Absorbance recorded at 254 nm. Gradient separation from 0.6 to 18 % acetonitrile with constant sodium phosphate concentration of 0.2 M. (see section 2.12 for details). Flow rate 1 $ml.min^{-1}$; total run time 25 min. Compounds identified as (1) CoASH; (2) succinyl-CoA; (3) acetyl-CoA; (4) acetoacetyl-CoA; (5) D(-)-3-hydroxybutyryl-CoA; (6) propionyl-CoA.

copolymers leaching from the ion-exchange resins following extensive use. The absorbance of the resulting stock phosphate buffer, measured at 254 nm, had decreased from 0.024 to a value less than 0.005, however, this was difficult to assess accurately. In comparison, Reiss and co-workers claim values less than 0.0006 absorbance units (1984) using this technique. The stock buffer was constantly recirculated through the three columns to prevent the recurrence of UV-absorbing contaminants which appear on standing.

Since the molar extinction coefficient of each CoA compound would be influenced by the composition of the eluant buffer/solvent at the point of detection (254 nm) it was necessary to compensate for these changes. Consequently, the molar extinction coefficient of each CoA compound was determined under the conditions in which it was detected. These values are summarised in Table 7.1 and closely parallel those obtained by King & Reiss (1985) using this technique.

Having demonstrated the practicality and sensitivity of reverse phase HPLC in conjunction with UV detection for the separation and quantification of the CoA compounds of interest in this study, the next section outlines the development of a protocol for the extraction of these compounds from continuous cultures of M. trichosporium OB3b.

COENZYME	E_M	E_{LC}
ACETOACETYL-CoA	15.4	14.8
ACETYL-CoA	15.4 (259)	14.3
CoASH	14.6	13.9
HYDROXYBUTYRYL-CoA	15.4	14.9
SUCCINYL-CoA	15.2	14.3
PROPIONYL-CoA	15.4	14.

Table 7.1 Comparison of Millimolar Absorption Coefficients

The millimolar absorption coefficient (E_M) for each CoA compound was determined at maximum wavelength (260 nm, unless otherwise stated) in phosphate buffer (0.2 M, pH 3.0). E_{LC} is the millimolar absorption coefficient determined under the conditions in which the compound was tested.

7.5.3 THE EXTRACTION AND QUANTIFICATION OF SHORT-CHAIN COA COMPOUNDS FROM M. TRICHOSPORIUM OB3B

One of the criticisms associated with many in vivo metabolite studies is generally aimed at the efficiency of the procedure used for the extraction of the metabolite(s) of interest. Classically disrepancies in the quantification of certain metabolites, especially adenine nucleotides (Harrison & Maitra, 1969), has been attributed to, in some cases, the method of extraction and, in particular, the time between sampling and quenching metabolic activity. This reflects essentially metabolite turnover as a result of changes in the metabolic state of the organism. As a prelude to this study it was necessary to assess the effect of sampling time on changes in the levels of each test compound.

The effect of a time delay following sample withdrawel from the fermenter and perchloric acid extraction of 6 CoA compounds is illustrated in Table 7.2. Clearly, there appeared to be no significant turnover of these compounds during the time course studied, and it was concluded that either cell metabolism was not significantly perturbed during this period or that the CoA compounds are turned over relatively slowly in this organism.

The optimal concentration of perchloric acid necessary to extract the CoA compounds from the cell material was determined to be 6 % (v/v), final concentration. Owing to the large dilution affects on each CoA compound by the growth medium present on extraction, it was not possible to quantitate each compound by direct HPLC analysis.

COENZYME	CONTENTS (pmol/mg dry weight) TIME (0 sec)	(30 sec)	(60 sec)
ACETOACETYL-CoA	10 +/- 0.6	9.6 +/- 0.9	10.9 +/- 0.7
ACETYL-CoA	194 +/- 8.6	182 +/- 6.9	188 +/- 12.7
CoASH	56 +/- 5.0	61 +/- 3.8	52 +/- 4.7
HYDROXYBUTYRYL-CoA	68 +/- 7.4	75 +/- 3.4	76 +/- 5.2
PROPIONYL-CoA	23 +/- 1.9	25 +/- 1.2	19 +/- 1.7
SUCCINYL-CoA	127 +/- 5.7	120 +/- 5.4	131 +/- 3.9

TABLE 7.3 The Effect of Delay After Sample Withdrawal Before Quenching on Metabolite Concentrations.

Samples (10 ml) were withdrawn from a continuous culture of M. trichosporium OB3b, growing on methanol (1.0 % (v/v)), into a syringe and held for intervals before quenching in perchloric acid. The samples were extracted, neutralised, and analysed for CoA compounds as described in section 2.12. Values reported represent triplicate analyses at each time interval.

Consequently, a concentration step was necessary, following perchloric acid extraction, in order to derive the maximum sensitivity from this technique. This was provided satisfactorily by freeze drying the neutralised perchloric acid extract, prior to sample analysis. This technique was used routinely for all sample preparation in this study. A typical chromatogram obtained following sample injection of a sample processed in this way is illustrated in Figure 7.7.

Metabolite loss during sample preparation was investigated in order to minimise any potential inaccuracy introduced into the quantifications of these compounds. For this experiment cultures were quenched in the normal way, followed by addition of a standard solution, containing a known concentration of each CoA compound of interest. The results are presented in Table 7.3. Losses of less than 10 % were noted and the final calculation of metabolite concentration was compensated to account for this. The effect of storage on either the neutralised perchloric acid extract or the freeze dried extract was not investigated during this study; instead the expedient neutralisation of the perchloric acid extract, followed by pH adjustment, sample concentration by freeze drying and analysis was performed at all times.

7.5.4 THE QUANTIFICATION OF COA COMPOUNDS DURING THE ACCUMULATION OF PHB IN METHANOL GROWN CONTINUOUS CULTURE OF M. TRICHOSPORIUM OB3B

For this experiment, the methanol feedstock concentration of a steady state continuous culture (D = 0.04 h^{-1}; D.O.T. of 40 %; biomass density 0.86 g (dry wt.) l-1) of M. trichosporium OB3b was increased

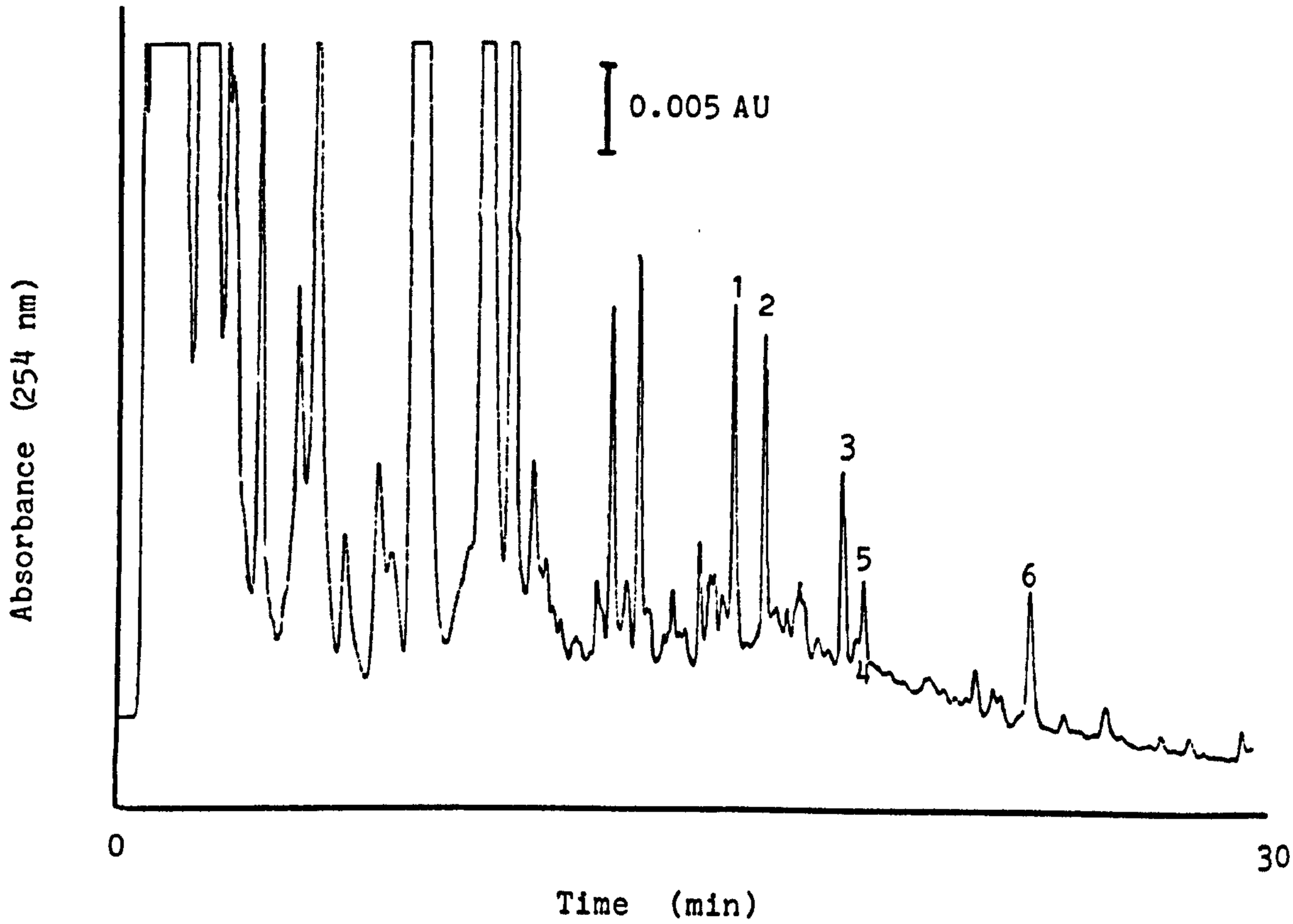


Figure 7.7 Chromatographic Separation of a Neutralised Perchloric Acid Extract of M. trichosporium OB3b, Grown in Continuous Culture on Methanol.

Identification of peaks is as follows: (1) CoASH; (2) succinyl-CoA; (3) acetyl-CoA; (4) acetoacetyl-CoA; (5) D(-)-3-hydroxybutyryl-CoA; (6) propionyl-CoA. Unknown peaks are not assigned. See section 2.12 for elution conditions and the method of sample preparation.

COENZYME	PERCENTAGE RECOVERY
ACETOACETYL-CoA	89 +/- 3
ACETYL-CoA	93 +/- 5
CoASH	85 +/- 7
HYDROXYBUTYRYL-CoA	90 +/- 7
PROPIONYL-CoA	91 +/- 4
SUCCINYL-CoA	92 +/- 4

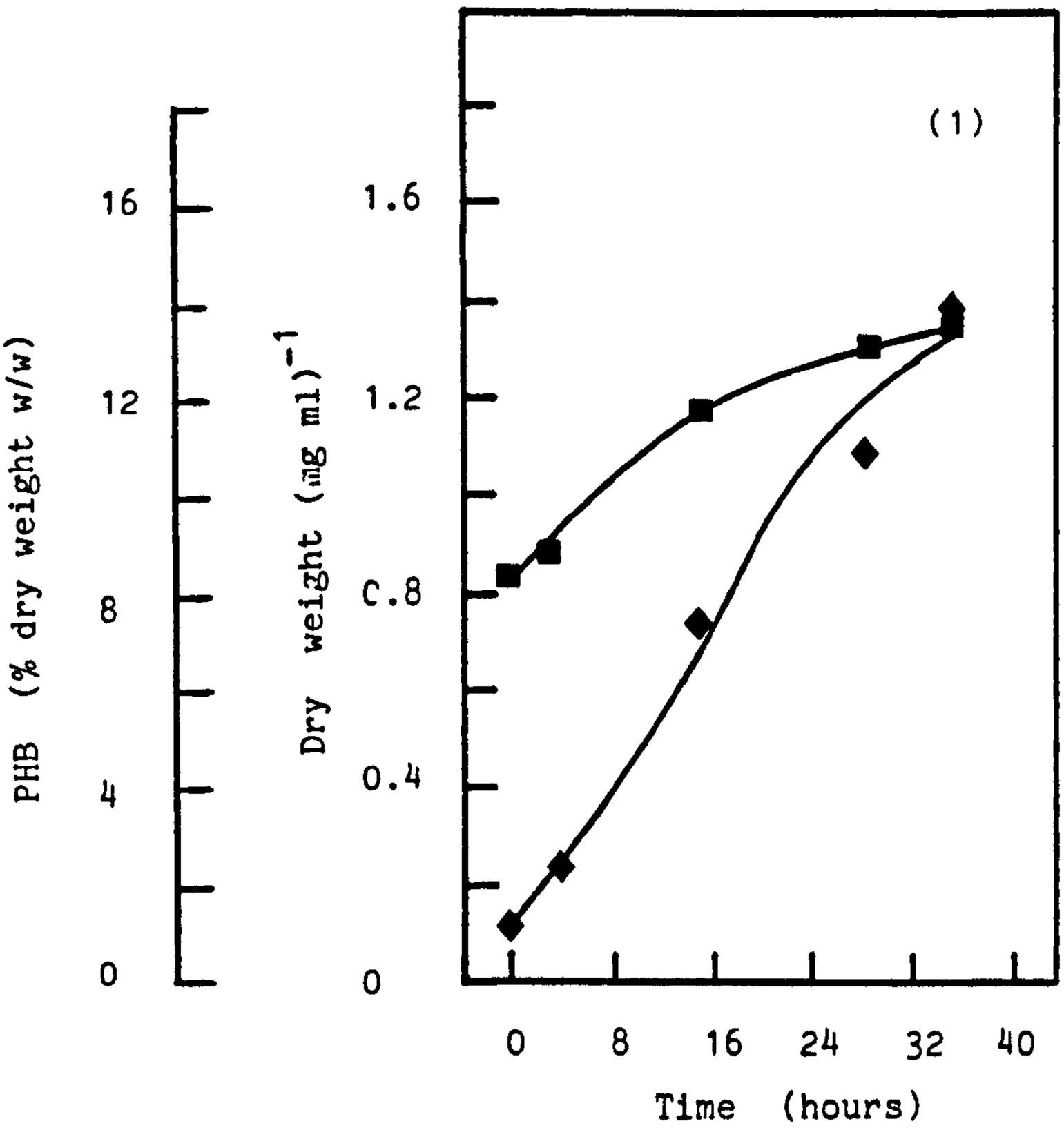
Table 7.2 Recovery of CoA Compounds From M. trichosporium OB3b

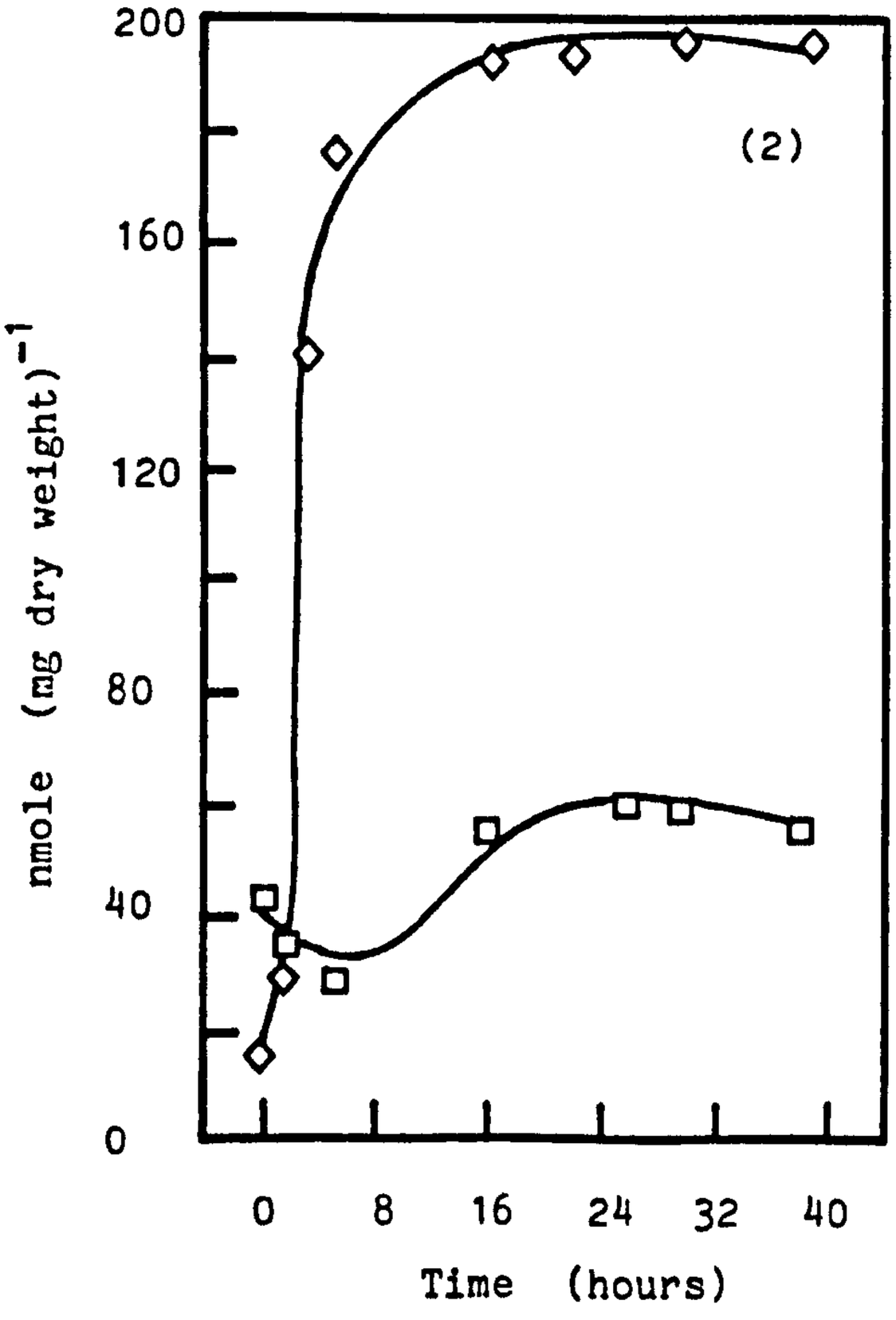
Samples (10 ml) were withdrawn from a continuous culture of M. trichosporium OB3b, growing on methanol (1.0 % (v/v)) and quenched in perchloric acid as described in section 2.12.1. An aliquot of a standard solution containing the CoA compounds listed above (0.5 μM of each compound) was added to each sample. Each sample was further processed as described in section 2.12.2. The percentage recovery of each compound represents the mean of six separate samples (corrected for the residual concentration of each compound in the cell) +/- SE.

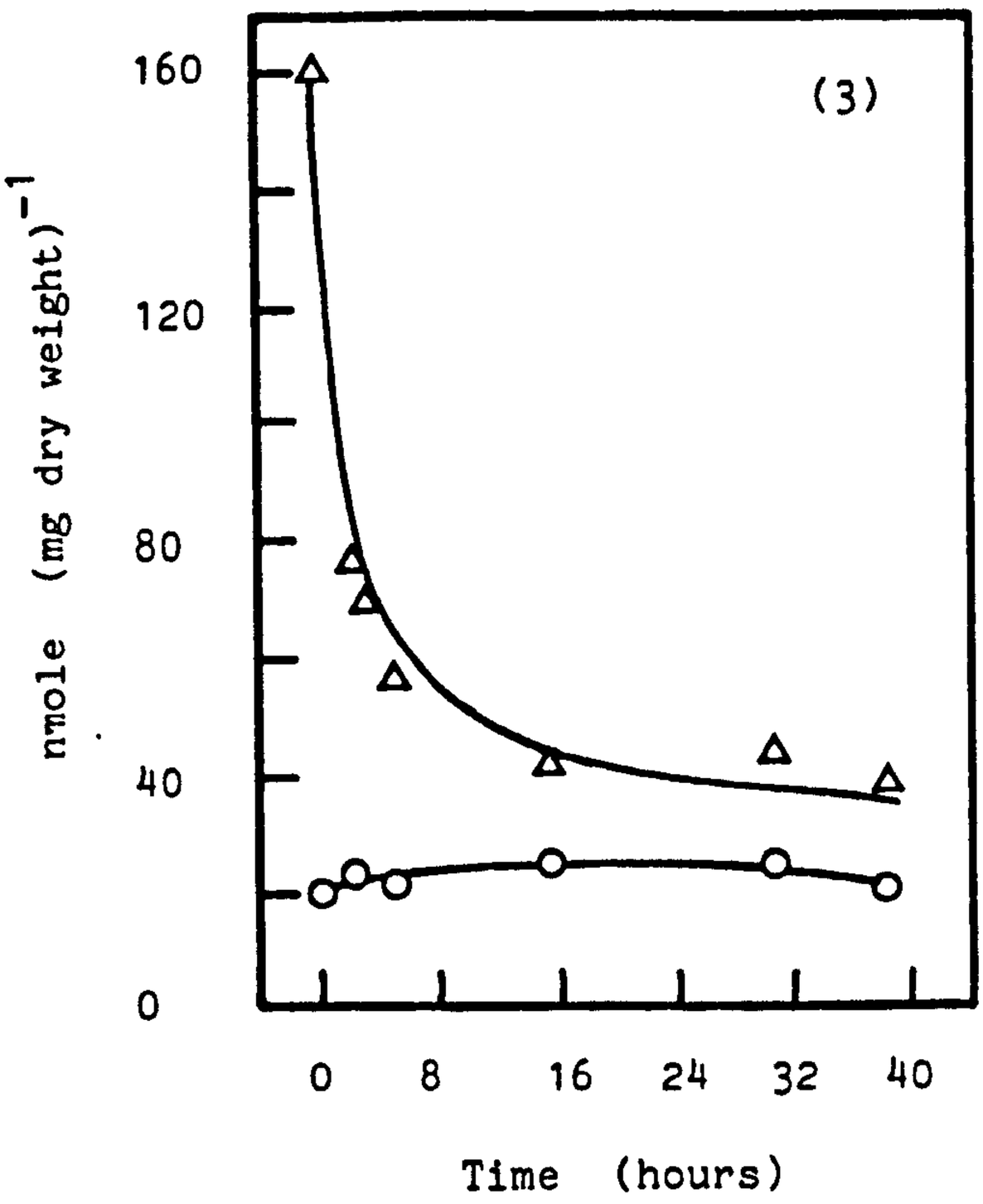
from 0.4 % (v/v) to 1 % (v/v). During this time, quantification of the CoA compounds described previously in this chapter, together with the PHB content (% dry weight) of the culture, was made.

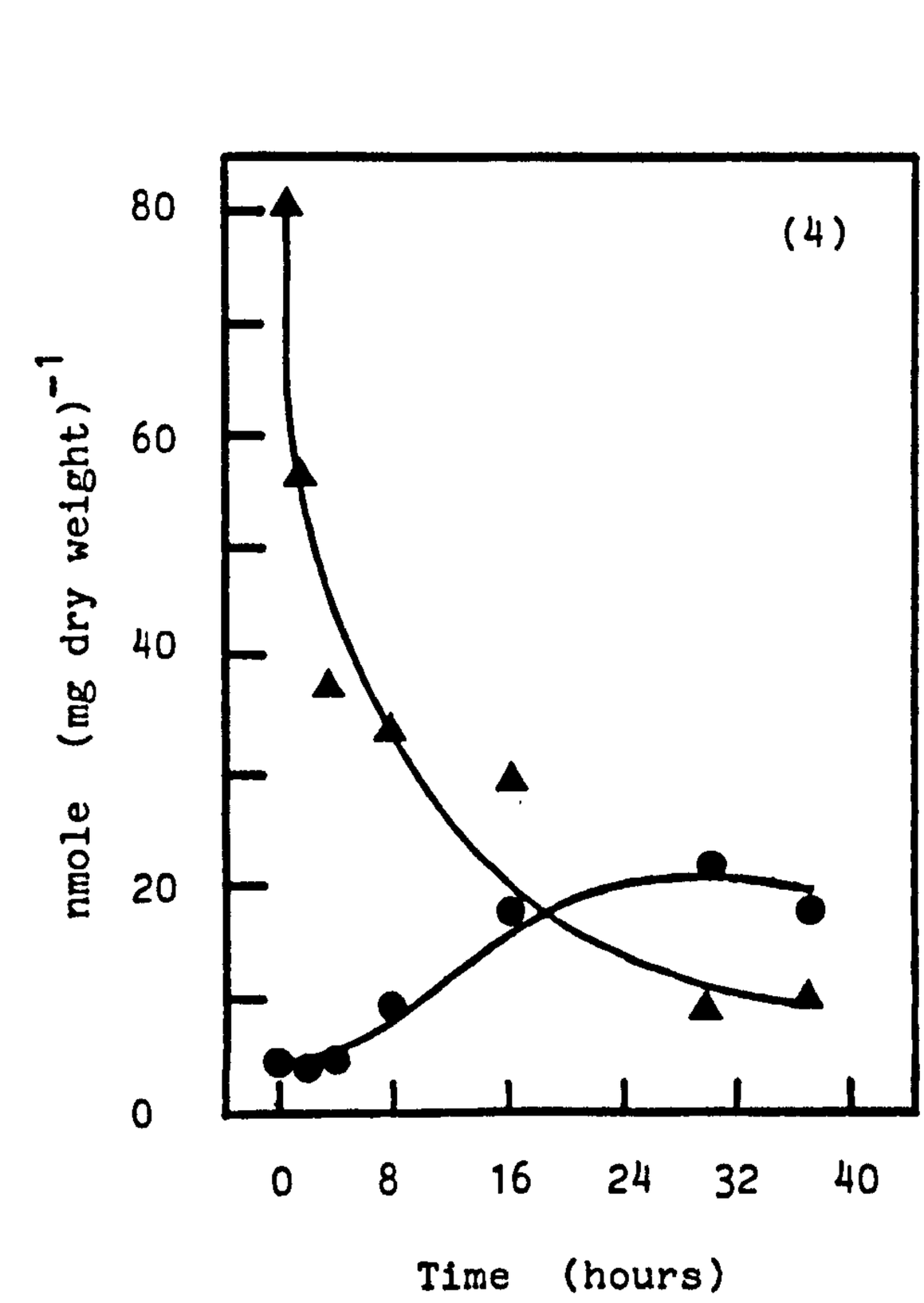
As observed previously, the dry weight and the PHB content of the culture increased during the transition from low to high methanol concentration (Fig. 7.8.1). Noticeably, the synthesis of PHB was closely paralled by a concomitant increase in metabolic flux through acetyl-CoA; the level of this metabolite increased over ten-fold during this period such that the steady state culture growing on 1.0 % (v/v) methanol contained 0.2 nmol acetyl-CoA (mg bacterial dry weight)$^{-1}$ (Figure 7.8.2). Of particular interest to this study, in terms of the regulation of beta-ketothiolase was the change in the acetyl-CoA/CoASH ratio during this period. The kinetic study on beta-ketothiolase, predicted that acetyl-CoA flux via PHB synthesis proceeded optimally at a high acetyl-CoA/CoASH ratio. In this experiment, the ratio of acetyl-CoA/CoASH changed from a value of 1:3 during steady state growth conditions on 0.4 % (v/v) methanol with a PHB content of 1.5 % (w/w) dry weight to a value of 3:1 during PHB synthesis. This change was predominantly accounted for by an increase in the intracellular level of acetyl-CoA since the level of CoASH increased only two-fold during this period.

Simultaneous measurement of D(-)-3-hydroxybutyryl-CoA and acetoacetyl-CoA during the transition from steady state growth on 0.4 % (v/v) to 1.0 % (v/v) methanol (Figure 7.8.3) indicated a 10-fold and 4-fold increase in the intracelluar levels of these compounds respectively.


(1)
PHB (% dry weight w/w)
Dry weight (mg ml)$^{-1}$
16
12
8
4
0
1.6
1.2
0.8
0.4
0
0
8
16
24
32
40
Time (hours)


(2)
nmole (mg dry weight)$^{-1}$
200
160
120
80
40
0
0
8
16
24
32
40
Time (hours)


(3)
160
120
80
40
0
nmole (mg dry weight)$^{-1}$
0
8
16
24
32
40
Time (hours)


(4)
80
60
40
20
0
nmole (mg dry weight)$^{-1}$
0
8
16
24
32
40
Time (hours)

In addition to the quantification of CoA compounds intimately associated with PHB metabolism, this study also highlighted the activity of the TCA cycle during PHB synthesis by the quantification of succinyl-CoA (Figure 7.8.4). The level of this compound increased almost 3-fold during the transition from 0.4 (v/v) to 1.0 (v/v) methanol. A steady state value of 0.12 nmol (mg culture dry weight)$^{-1}$ was obtained during steady state culture on 1.0 % (v/v) methanol. An unexpected product of this study was the detection of another CoA compound, which was identified as propionyl-CoA. The evidence to support this discovery was based upon the coelution of a standard solution of propionyl-CoA with the peak obtained from the perchloric acid extracts from this organism. In order to determine whether there were any other materials coeluting with propionyl-CoA or indeed with any other compounds in this study, an aliquot of a control extract was treated with base to hydrolyse the acyl-CoAs present (Corkey et al., 1981). The extract pH was adjusted to 12.2 using potassium hydroxide and the extract was heated at 55^{o} C for 10 min, after which the pH was adjusted to 3.2 with perchloric acid. Analysis of the resulting sample indicated that no other materials coeluted with propionyl-CoA or any other compound studied in this section.

The discovery of propionyl-CoA provides the first substantiated evidence of the involvement of this compound in methanotrophic metabolism, the implications of which will be discussed in the next section (section 7.6). Another interesting aspect of this study was the pattern of accumulation/disappearance of propionyl-CoA. During the early phase of PHB synthesis the level of this compound increased

28-fold, to a maximum of 0.14 nmol (mg dry weight)$^{-1}$. This concentration was not maintained, since shortly thereafter it decreased to give a steady state level of 0.02 nmol (mg dry weight)$^{-1}$. One possible explanation emerge of this occurrance was that propionyl-CoA metabolism was limited by the activity of another enzyme on the pathway associated with its metabolism.

7.5.5 THE QUANTIFICATION OF COA COMPOUNDS DURING THE MOBILISATION OF PHB IN METHANOL GROWN CONTINUOUS CULTURES OF M. TRICHOSPORIUM OB3B

The kinetic data obtained from the study on beta-ketothiolase and acetoacetyl-CoA reductase from M. trichosporium OB3b suggested that PHB mobilisation via acetyl-CoA was dependant upon the intracellular concentration of CoASH, acetoacetyl-CoA and acetyl-CoA. Evidence to substantiate these proposals was investigated in this section.

For this experiment, a steady state continuous culture of M. trichosporium OB3b, grown on 1 % (v/v) methanol was subject to a decrease in the inlet concentration of methanol to 0.4 % (v/v). The dry weight of the culture and its PHB content fell from a steady state value of 1.52 g (dry weight) $1^{-1}$and 14.4 % (w/w) (dry weight) respectively, to 0.78 g (dry weight) 1^{-1} and 2.6 % (w/w)(dry weight) (Figure 7.9.1). During this period the ratio of acetyl-CoA/CoASH changed from 3:1 to 1:2 (Figure 7.9.2); whereas the level of acetoacetyl-CoA increased more than six-fold (Figure 7.9.3). Although it is difficult to rationalise the direct significance of this data in the absence of actual intracellular concentrations of these metabolites, the trends in metabolite accumulation under these

Figure 7.9 Effect of Transition From Methanol Excess (1.0 % v/v) to Methanol Limitation (0.4 % v/v) on In Vivo Metabolite Concentration in Continuous Culture of M. trichosporium OB3b.

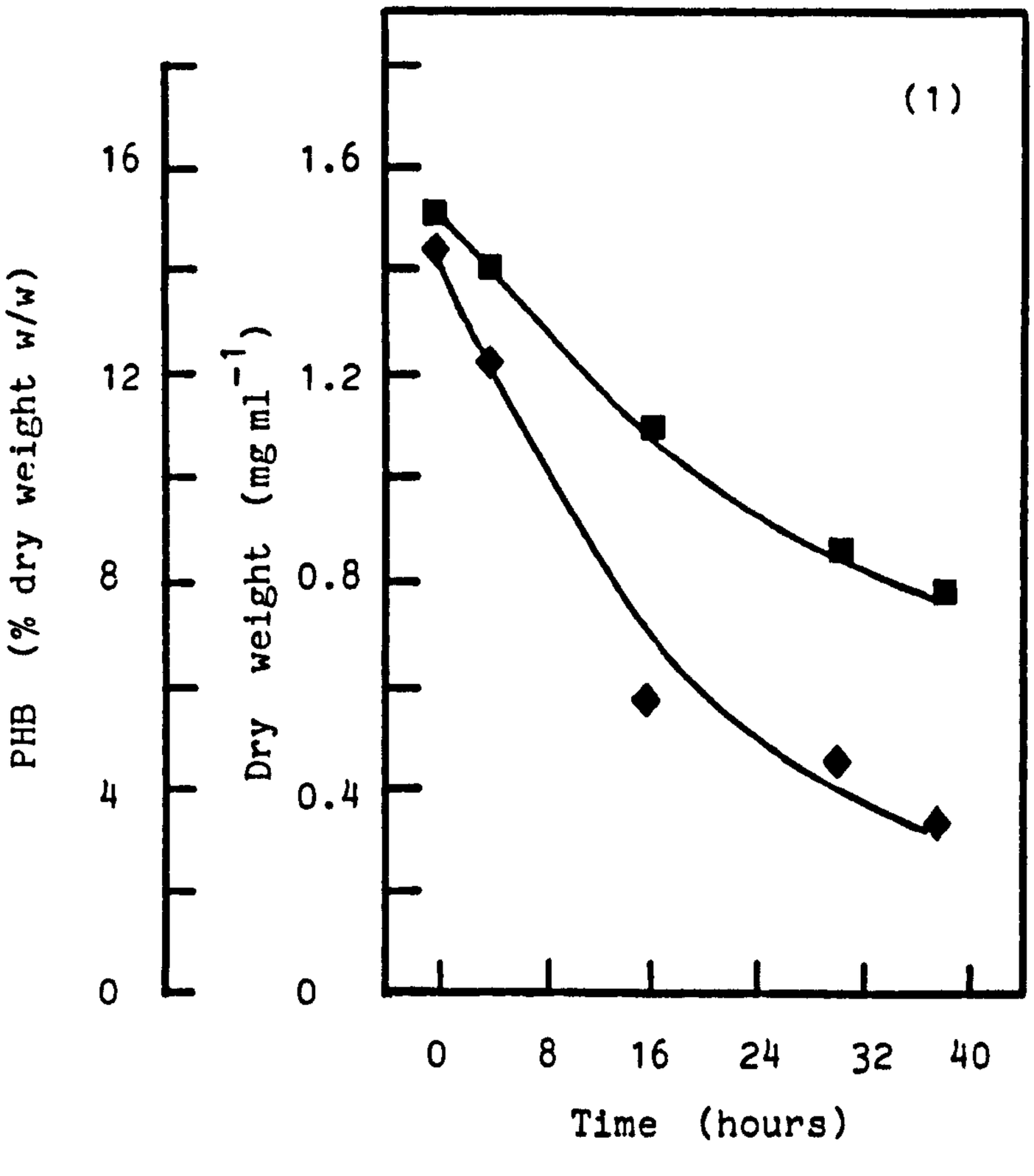
The experimental details are contained in the text.

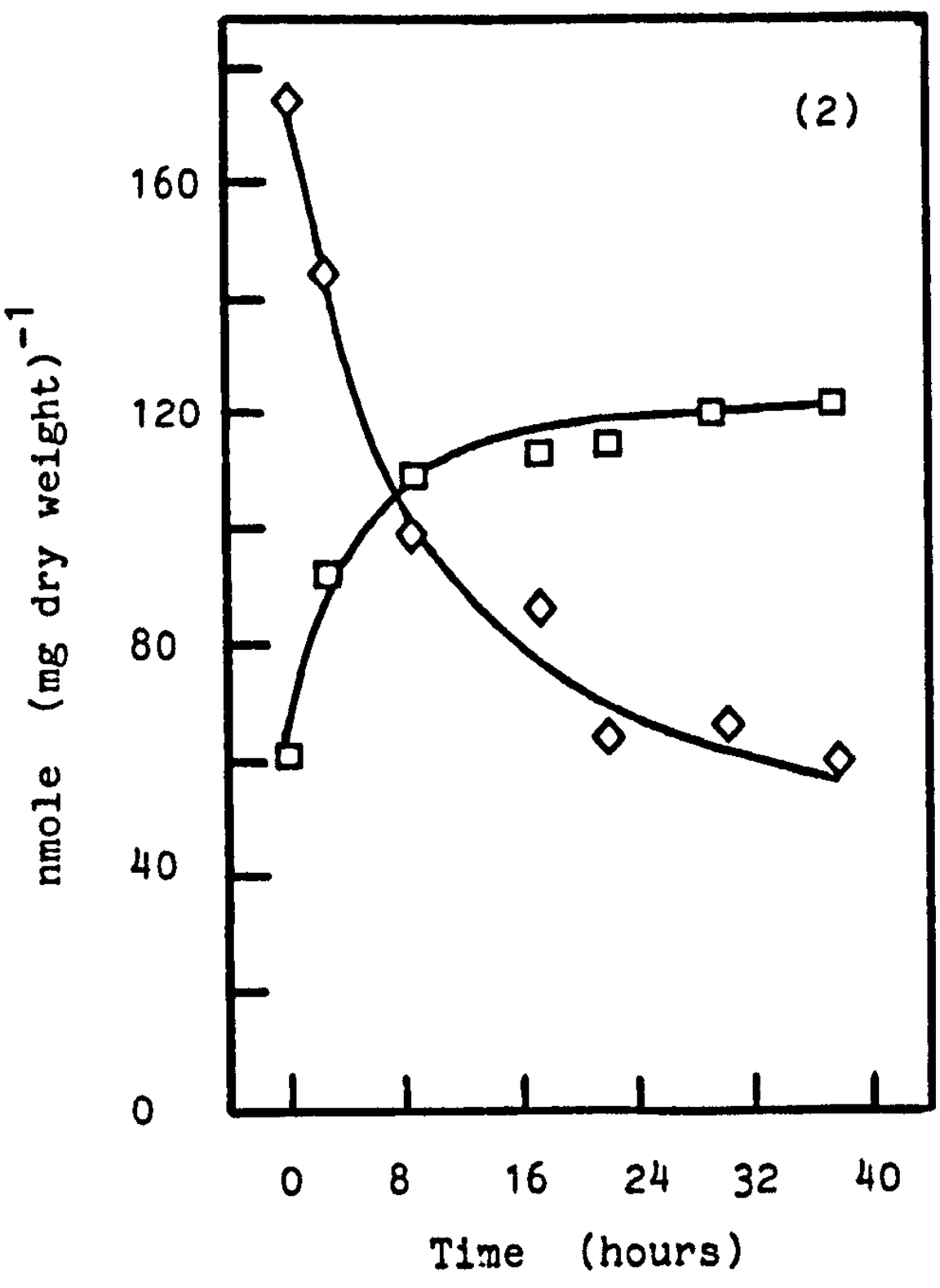
(1) (◆) PHB content; (■) bacterial dry weight.

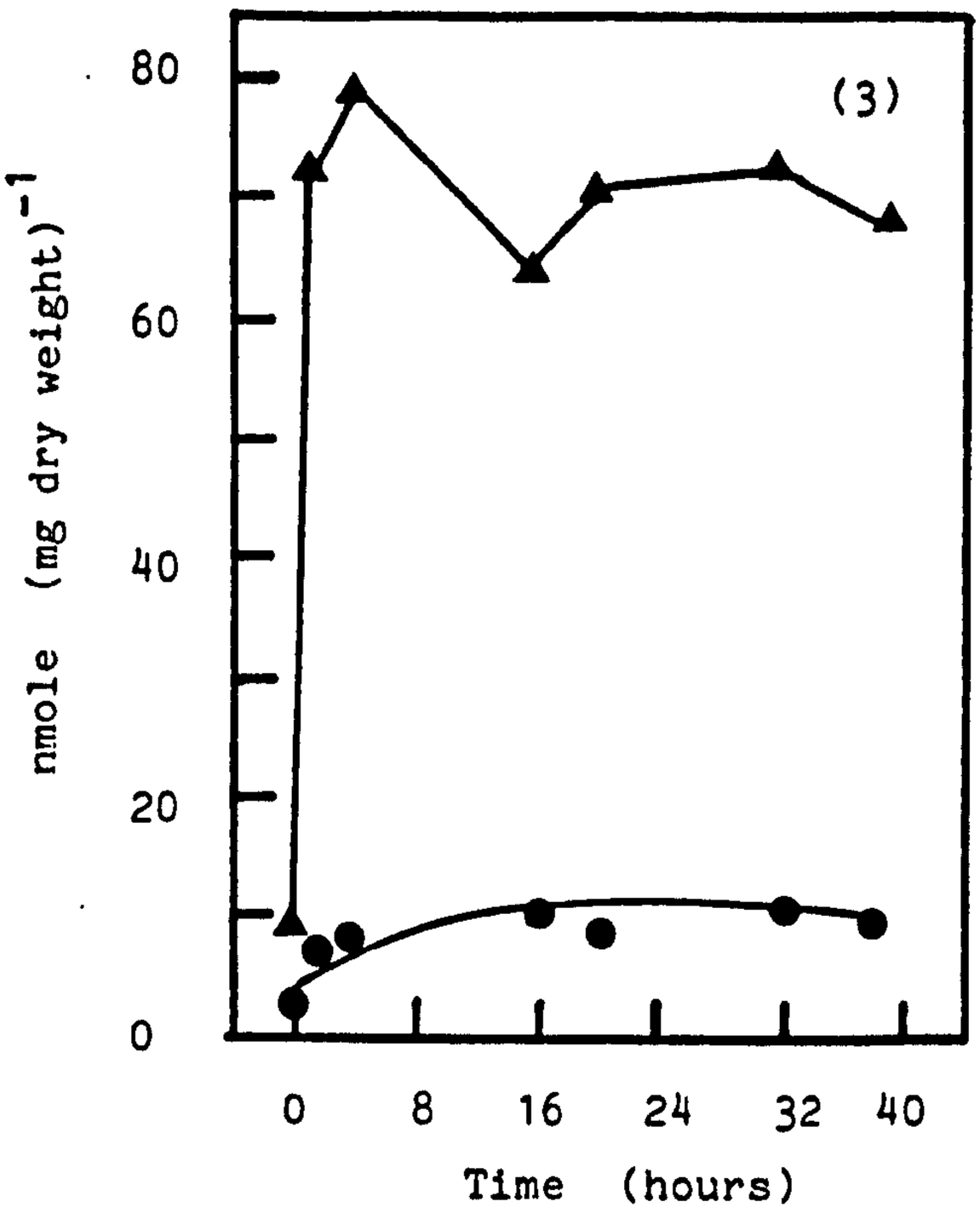
(2) (◇) Acetyl-CoA; (□) CoASH.

(3) (▲) D(-)-3-Hydroxybutyryl-CoA; (●) acetoacetyl-CoA.

(4) (△) Succinyl-CoA; (○) propionyl-CoA.


(1)
PHB (% dry weight w/w)
Dry weight (mg ml^{-1})
16
12
8
4
0
1.6
1.2
0.8
0.4
0
0
8
16
24
32
40
Time (hours)


(2)
nmole (mg dry weight)$^{-1}$
160
120
80
40
0
0
8
16
24
32
40
Time (hours)


(3)
nmole (mg dry weight)$^{-1}$
80
60
40
20
0
0
8
16
24
32
40
Time (hours)

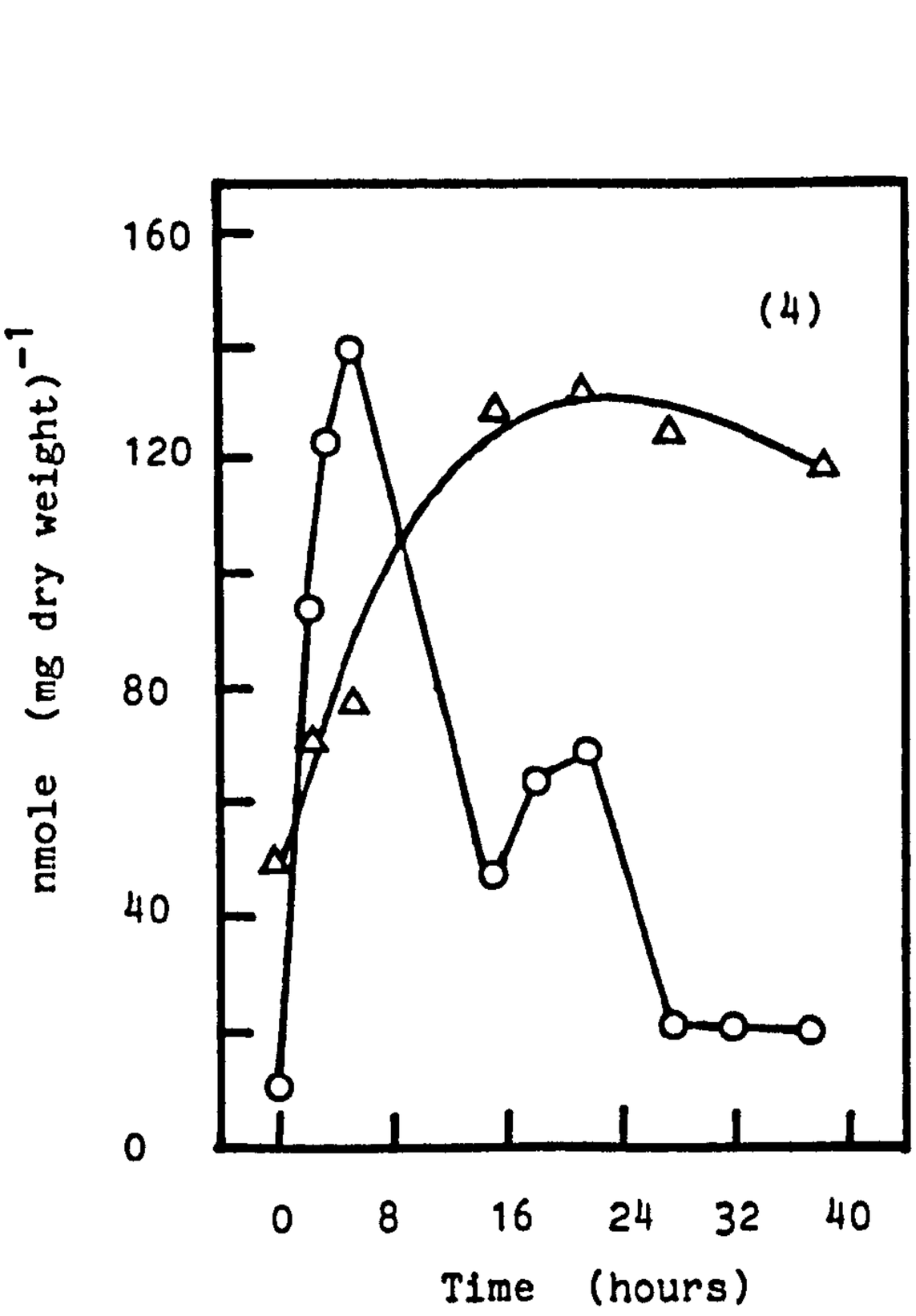

(4)
nmole (mg dry weight)$^{-1}$
160
120
80
40
0
0
8
16
24
32
40
Time (hours)

Figure 7.8 Effect of Transition from Methanol Limitation (0.4 % v/v) to Methanol Excess (1.0 % v/v) on In Vivo Metabolite Concentration in a Continuous Culture of M. trichosporium OB3b.

The experimental details are contained in the text and in section 2.12

(1) (◆) PHB content; (■) bacterial dry weight.

(2) (◇) Acetyl-CoA; (□) CoASH.

(3) (▲) D(-)-3-Hyroxybutyryl-CoA; (●) acetoacetyl-CoA.

(4) (△) Succinyl-CoA; (○) propionyl-CoA.

circumstances can be related to <u>in vitro</u> enzyme kinetic data; this is highlighted below.

As one might expect, the intracellular level of D(-)-3-hydroxybutyryl-CoA decreased with the cessation of PHB synthesis and the start of PHB mobilisation. This again highlights its constitutive importance as a precursor of this material. Interestingly, the concentration of propionyl-CoA did not change during the mobilisation of PHB, which contrasted to its accumulation during PHB synthesis (Figure 7.9.4). One possible explanation for this occurrence is that the pathway leading from the mobilisation of PHB is distinct from that used during its synthesis; this point is discussed in the next section in the wider context of the intermediary metabolism of this organism.

7.6 DISCUSSION

An understanding of the integrated control of metabolism has been largely provided by the advances in enzymology during the last twenty years. However, the postulates interrelating metabolite concentration and control, derived from work on purified enzymes, are infrequently verified *in vivo*. Attempts to redress this balance have been sporadic (Lowry *et al.*, 1971; Bowien *et al.*, 1974; Cook *et al.*, 1976) and probably reflect the difficulty of growing well-known strains of bacteria at high densities under reliable, defined conditions (or validly harvesting a sample) and the problem in attaining the necessary sensitivity with the assay methods used.

No complete study on the integrated control of PHB metabolism, has been detailed for any organism studied to date. Whereas kinetic studies on enzymes associated with PHB metabolism have been fairly extensive (Senior & Dawes, 1973; Oeding & Schlegel, 1973; Nishimura *et al.*, 1978; Saito *et al.*, 1977; Nakada *et al.*, 1981), there is little or no *in vivo* metabolite data to corroborate the information obtained in these studies.

Limited *in vivo* metabolite studies on a mutant of *A. eutrophus* H16, defective in its ability to produce PHB, suggested that pyruvate, malate and citrate were excreted under conditions characterised by PHB production in the wild type organism (Cook & Schlegel, 1978). The relationship between the accumulation of these metabolites and the regulation of PHB metabolism was not, however, assessed. In the case

of A. beijerinkii, metabolite studies were restricted to measurement of NADH/NAD ratios at the onset of PHB synthesis (Jackson & Dawes, 1976). This study served to demonstrate the role of PHB as an electron sink for the re-oxidation of NADH. However, the study was not extended to include the measurement of NADH/NAD ratios during polymer mobilisation and therefore, the significance of this redox couple in the regulation of PHB mobilisation could not be assessed.

The results of this chapter, and indeed, those of the previous results chapters, combine to represent the most complete description on the integrated control of PHB metabolism in any organism studied to date. A significant contribution to the success of this study was the development of a method for the simultaneous quantification of the CoA compounds, previously identified as effectors of enzymes (acetoacetyl-CoA reductase and beta-ketothiolase) associated with polymer synthesis/mobilisation. Reverse phase HPLC, in conjunction with UV detection, provided an extremely rapid and sensitive technique for the separation and quantification of the six compounds of interest in this study. Laborious, multi-step metabolite analysis was eradicated by this technique and the decomposition of each of the CoASH compounds was minimised, since each were analysed under conditions optimal for their stability (King & Reiss, 1985). In addition to monitoring carbon flux through the CoA intermediates associated with polymer metabolism the technique afforded information on the activity of the TCA cycle, via quantification of succinyl-CoA.

Since a good deal of information exists to describe the interelationship between the physiological conditions responsible for PHB metabolism in both A. eutrophus H16 (Oeding & Schlegel, 1973) and A. beijerinkii (Senior & Dawes, 1973; Jackson & Dawes, 1976; Ward et al., 1977), and the potential effect of metabolite accumulation on the regulation of intermediary and polymer metabolism, the following discussion compares this information with the data obtained on the organism in this study.

A. beijerinkii was shown to accumulate large quantities of PHB during oxygen-limited growth (Senior & Dawes, 1972). Oxygen limitation was postulated to produce conditions which yielded a high intracellular NAD(P)H/NAD(P) ratio. This was based on the assumption that the pyridine nucleotides were not reoxidised under these conditions. Since citrate synthase (inhibited by NADH) and isocitrate dehydrogenase (inhibited by NAD(P)H) would be inhibited under these conditions, this, it was thought, would effectively decrease the rate of acetyl-CoA metabolism via the TCA cycle. Accumulation of acetyl-CoA would result in its metabolism via D(-)-3-hydroxybutyryl-CoA (with the concomitant reoxidation of NAD(P)H) and subsequent PHB formation. Conversely, on the relaxation of oxygen limitation, it was suggested that this process is reversed and that polymer synthesis ceases when acetyl-CoA is again oxidised via the TCA cycle.

The mechanism for the regulation of PHB metabolism in A. beijerinkii was based primarily on in vitro enzyme kinetic data (Senior & Dawes, 1973). This data was rationalised as follows: under conditions of unrestricted growth, in the presence of excess oxygen

(i.e. no polymer synthesis), the steady state concentration of CoASH was thought to be high as a consequence of citrate synthase activity. A high CoASH/acetyl-CoA ratio would not only ensure a low rate of acetoacetyl-CoA synthesis, through the sub-optimum concentration of acetyl-CoA for beta-ketothiolase activity (K_m of beta-ketothiolase for acetyl-CoA = 0.9 mM), but that PHB synthesis was restricted by CoASH inhibition of this enzyme. During oxygen-limited growth, however, NADH inhibition of citrate synthase would result in an increase in the acetyl-CoA/CoASH ratio, a decrease in the inhibition of beta-ketothiolase, and metabolism via acetoacetyl-CoA.

In the absence of information on the enzyme(s) associated with the initial attack on the PHB molecule, the regulation of polymer mobilisation and degradation was thought likely to occur through the pyridine nucleotide-mediated oxidation of monomeric D(-)-3-hydroxybutyrate by 3-HBD. This enzyme was regulated by the NADH/NAD couple in addition to metabolic intermediates or precursors of the TCA cycle (pyruvate and 2-oxoglutarate).

Kinetic studies on beta-ketothiolase from A. eutrophus H16 (Oeding & Schlegel, 1973), together with inhibition studies on other enzymes in the pathway of PHB metabolism, suggested that a similar control mechanism to that outlined in A. beijerinkii, also operated in this organism. Nitrogen limited growth was primarily responsible for PHB synthesis, although previous studies (Morinaga et al., 1978) indicated that oxygen limited growth could similarly induce polymer synthesis.

In both cases highlighted above, polymer metabolism appeared to be regulated to a large extent by the activity of the TCA cycle. The information elucidated from in vivo metabolite studies on chemostat cultures of M. trichosporium OB3b, however, suggested a distinctly different mechanism for the regulation of polymer metabolism. Consequently, a large proportion of the following discussion elaborates the information on which this conclusion is based.

In order to propose a mechanism by which M. trichosporium OB3b regulates the partition of acetyl-CoA between the TCA cycle, via citrate synthase, and polymer formation via beta-ketothiolase, it is necessary to account for the physiological conditions which result in PHB metabolism and relate those to the enzymological data and in vivo metabolite information obtained from this organism. Chemostat experiments with M. trichosporium OB3B revealed that nitrogen limitation, rather than oxygen limitation, was probably responsible for PHB synthesis. Under these conditions the organism was capable of accumulating up to 20 % of its dry weight as PHB. The study was not exhaustive, however, and does not exclude the possibility that any one of a number of limitations on growth such as phosphate, sulphate, magnesium or potassium may result in PHB synthesis in this organism.

In common with all Type II methanotrophs examined to date, M. trichosporium OB3b assimilates nitrogen (ammonia) via the GS/GOGAT pathway (Shishkina & Trotsenko, 1979; Murrell & Dalton 1983a). During nitrogen-limited growth the imbalance between carbon and nitrogen assimilation would probably result in the accumulation of TCA cycle intermediates. This, it is proposed, results from a decrease in

metabolism of 2-oxoglutarate and glutamine (product of nitrogen assimilation) via the enzyme complex GOGAT. The evidence to substantiate this proposal was provided, indirectly, by in vivo quantification of succinyl-CoA and acetyl-CoA during the early phase of polymer synthesis. The concentration of these compounds increased by 3 and 10-fold respectively during this period and mirrors what one might expect if there was a decrease in the rate of 2-oxoglutarate metabolism via glutamate.

Elevated intracellular concentrations of succinyl-CoA during PHB accumulation in M. trichosporium OB3b suggested that the TCA cycle was fully operational during this period. Feedback inhibition of the type described above for both A. beijerinkii or A. eutrophus H16 would have resulted in a decrease in the intracellular concentration of succinyl-CoA. The conclusion that PHB synthesis in M. trichosporium OB3b is a result of overspill metabolism from the TCA cycle seems the most likely interpretation of this information.

The postulates outlining the role and regulation of beta-ketothiolase (section 5.3) in M. trichosporium OB3b were verified following in vivo metabolite studies. This demonstrated that during PHB synthesis, the ratio of acetyl-CoA/CoA switches from 1:3 to 3:1. Although these results are open to interpretation in the absence of absolute concentrations of these compounds, they nevertheless suggest that a high acetyl-CoA/CoA ratio, necessary for the expression of beta-ketothiolase activity, is provided during polymer synthesis.

Quantification of acetoacetyl-CoA, during PHB synthesis, indicated that its concentration was relatively low in comparison to the steady state concentration of acetyl-CoA (one-tenth the steady state concentration of acetyl-CoA). This parallels the kinetic studies on acetoacetyl-CoA reductase (section 6.2), which suggested that the efficient metabolism of this molecule was necessary, in order to prevent substrate inhibition of the enzyme and a decrease in the rate of PHB synthesis. Although, PHB synthase was not examined in the course of this thesis, in vivo quantification of D(-)-3-hydroxybutyryl-CoA during polymer synthesis suggested that the polymerisation of this molecule was the rate limiting step in this pathway as it was seen to accumulate.

An interesting and yet unresolved consequence of the in vivo metabolite study was the first report on the detection of propionyl-CoA in a methanotroph. Since the pathway outlined by Shimizu (1984) to describe the ICL^- pathway in these organisms incorporates a scheme for metabolism via propionyl-CoA, it is tempting to suggest that the detection of this molecule might account for the operation of this pathway in this organism. Certainly recent work (Richard Watkins, personal communication) substantiates this proposal since all ICL^+ organisms tested did not produce propionyl-CoA, whereas the corollary is true of all the ICL^- organisms examined. It should be emphasised, however, that this information alone is insufficient to substantiate Shimizu's claims. Nevertheless, it provides a significant pointer to further study in this area of methanotrophic/methylotrophic intermediary metabolism.

The factors responsible for PHB mobilisation were not resolved in this study. In vitro inhibition studies conducted on 3-HBD, suggested that the NADH/NAD ratio might, in part, account for this. By a reversal of nitrogen limition on growth, one might expect a shift in the NADH/NAD ratio as nitrogen is again assimilated by the cell. The concentration of TCA cycle intermediates might be expected to fall as a consequence, and indeed this is verified by this study. The rise in acetoacetyl-CoA is in line with that expected for this compound in order to prevent a futile cycle of reduction to D(-)-3-hydroxybutyryl-CoA. No doubt, as the balance of NADP/NADPH is shifted in favour of the former compound, this too, contributes to a reduction in the activity of acetoacetyl-CoA reductase. In order to complete this study and confirm the regulation of polymer mobilisation (section 3.3), it would be essential to measure NAD(P)H/NADP ratios during polymer metabolism.

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